

**The in vitro effects of
cigarette smoke on
models of epithelial injury.**

by

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DECLARATION

I declare that this Thesis was composed by myself and that the experimental studies reported are my own. None of the work included in this Thesis has been submitted for any other degree or professional qualification.

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ABSTRACT

Cigarette smoking has been shown to increase air space epithelial permeability, which is thought to be an early indication of air space epithelial injury. A large number of toxic compounds, in particular oxygen radicals, are produced by cigarette smoke, and it may be the imbalance between these and the intra- and extracellular antioxidants present in the lungs which is critical in inducing lung injury.

Both superoxide dismutase and catalase have been shown to increase intracellularly in hamsters exposed to cigarette smoke. Reduced glutathione, another important antioxidant, is also present in increased concentrations in erythrocytes and in the lung epithelial lining fluid of cigarette smokers. This may be in response to the increased radical burden in smoking in an attempt to confer protection to the cells in the airway and alveoli of the lung. However the acute effects of smoking have received less attention.

In this study the attachment, detachment and proliferation of the A549 human alveolar epithelial cell line was examined in an *in vitro* model of cell injury and the effects of exposure to cigarette smoke and its condensates on these assays of epithelial function were assessed. In addition a comparison of the effects of cigarette smoke on the bronchial cell line, BEA-2B or freshly isolated rat type II cells, and the protective effects of the antioxidants, were also studied.

Whole and vapour phase cigarette smoke decreased ^{51}Cr -labelled A549 epithelial cell attachment in a dose-dependent manner. The condensates prepared from both types of cigarette smoke increased cell detachment, and decreased cell proliferation, as assessed by the uptake of $[^3\text{H}]$ thymidine by A549 epithelial cells. Both freshly isolated rat type II

alveolar epithelial cells and cells of the bronchial epithelial cell line, BEA-2B, showed enhanced susceptibility to the injurious effects of cigarette smoke condensates when compared with the A549 epithelial cell line. The enzymatic antioxidants superoxide dismutase and catalase, when present extracellularly, gave partial protection against the effects of cigarette smoke or condensate exposure on cell attachment, detachment and proliferation. However extracellular reduced glutathione, in physiological concentrations ($400\mu\text{M}$), conferred total protection against cigarette smoke or condensate induced cell injury. Depletion of intracellular reduced glutathione also enhanced the epithelial cell detachment produced by cigarette smoke condensates. The oxidant hydrogen peroxide also decreased A549 epithelial cell attachment and increased cell detachment, which was prevented by the addition of reduced glutathione.

To examine possible mechanisms of cigarette smoke induced changes in epithelial cell attachment and detachment the role of the cell surface adhesion molecules, and the cell cytoskeleton and changes in intracellular signalling were studied. Both the A549 and the BEA-2B cell lines stained positive for integrins for the extracellular matrix. Cigarette smoke exposure of these cells did not effect integrin expression, but this does not rule out changes in integrin function. Surface adhesion molecule function is cationic dependent and chelation of the cations was found to cause epithelial detachment.

Activation of protein kinase C or increasing intracellular calcium also increased epithelial detachment, whereas inhibition of these effects reduced the level of epithelial cell injury produced by cigarette smoke condensate. This suggests that both protein kinase C and intracellular calcium play a role in cigarette smoke condensate induced epithelial cell

injury. The cytoskeleton is also involved in epithelial cell detachment since disruption of the microfilaments of the epithelial cell cytoskeleton with cytochalasin also lead to increased detachment

These studies indicate that exposure of epithelial cells to cigarette smoke or its condensates in vitro causes cell injury. The mechanism of this effect appears to be oxidant mediated and may be initiated through a variety of intracellular signalling pathways.

| | |
|--------|--|
| CCO | cigarette smoke condensate |
| DMSO | dimethyl sulfoxide |
| DNase | deoxyribonuclease |
| EDTA | ethylenediaminetetraacetic acid |
| EGTA | ethyleneglycolbis(2-aminoacetic acid) |
| ELF | epithelial lining fluid |
| FCs | fetal calf serum |
| IMLP | 1-methyl-2-imidazolidinone |
| GSH | reduced glutathione |
| HBS | Hanks buffered salt solution |
| LPB | lipopolysaccharide |
| mAb | monoclonal antibody |
| MEM | minimal essential medium |
| PBS | phosphate buffered saline |
| PDEc | phorbol 12, 13-dibutyrate |
| PEG | poly(ethylene glycol) |
| PMA | phorbol 12-myristate 13-acetate |
| PMA-ME | phorbol 12-myristate 13-acetate 4-O-methyl-ether |
| SCG | soluble cigarette smoke |
| SOD | superoxide dismutase |

ABBREVIATIONS

| | |
|-----------------|--|
| α -1-PI: | alpha-1-proteinase inhibitor |
| BAL: | bronchoalveolar lavage |
| BALF: | bronchoalveolar lavage fluid |
| BSA: | bovine serum albumin |
| BSO: | buthionine sulfoxamine |
| CMEM: | minimal essential medium containing 10% fetal calf serum |
| CSC: | cigarette smoke condensate |
| DMSO: | dimethyl sulphoxide |
| DNase: | deoxyribonuclease |
| EDTA: | ethylenediaminetetraacetic acid |
| EGTA: | ethyleneglycoltetraacetic acid |
| ELF: | epithelial lining fluid |
| FCS: | fetal calf serum |
| fMLP: | f met leu phe |
| GSH: | reduced glutathione |
| HBSS: | Hanks buffered salt solution |
| LPS: | lipopolysaccharide |
| mAb: | monoclonal antibody |
| MEM: | minimal essential medium |
| PBS: | phosphate buffered saline |
| PDBu: | phorbol 12,13-dibutyrate |
| PEG: | monomethyloxypolyethylene glycol |
| PMA: | phorbol 12-myristate 13-acetate |
| PMA-4ME: | phorbol 12-myristate 13-acetate 4-0-methyl-ether |
| SCS: | soluble cigarette smoke |
| SOD: | superoxide dismutase |

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CHAPTER 1

INTRODUCTION

1.1 HISTORY OF SMOKING AND DISEASE

Tobacco was first introduced into Europe at the end of the fifteenth century. It was first used as a food and then as a medicine. It was not until the sixteenth century that it was used as a recreational drug. The use of tobacco as a recreational drug was first introduced into Europe by the Spaniards in the sixteenth century. It was not until the eighteenth century that it became a popular habit in Europe. The use of tobacco as a recreational drug was first introduced into Europe by the Spaniards in the sixteenth century. It was not until the eighteenth century that it became a popular habit in Europe.

CHAPTER 1

INTRODUCTION

The history of smoking and disease is a long and complex one. It is a story of the human race's relationship with a plant that has both good and bad effects. The story begins in the Americas, where the indigenous people have been smoking tobacco for thousands of years. The Spaniards brought tobacco to Europe in the sixteenth century, and it quickly became a popular habit. The use of tobacco as a recreational drug was first introduced into Europe by the Spaniards in the sixteenth century. It was not until the eighteenth century that it became a popular habit in Europe.

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CHAPTER 1

INTRODUCTION

1.1 HISTORY OF SMOKING AND DISEASE

Tobacco was first introduced into Europe at the end of the fifteenth century, and was considered to be beneficial and indeed medicinal! However it was only a century later, and almost four centuries before this study, that the noxious effects of tobacco were being recognised. It is fitting that a Scottish King, James VI of Scotland and I of England, imposed a heavy import tax on tobacco in 1604. He concluded in his Counterblaste to Tobacco that smoking is:

"a custome loathsome to the Eye, hateful to the Nose, harmful to the Braine, dangerous to the Lungs, and, in the black stinking Fume thereof, nearest resembling the horrible Stygian Smoke of the pit that is bottomless" (Reviewed in Tobacco fashions, 1957).

With this statement James was far ahead of his time since we now know that cigarette smoking is an important factor in the pathogenesis of cerebrovascular and ischaemic heart disease, cancer particularly lung and oesophageal cancer and chronic obstructive pulmonary disease (COPD) (Mason *et al*, 1985).

It was not until the end of the nineteenth, and into the beginning of the twentieth centuries, that cigarette smoking became fashionable for the masses and consumption rapidly increased (Royal College of Physicians of London, 1962). This trend in increased consumption continued well into this century and has only recently started to reverse (Lee *et al*, 1990). With this increase in consumption came an increase in lung disease. In

1938, a longitudinal study of over 6,000 men with verified smoking histories reported an increased correlation between smoking habit and longevity (Pearl, 1938). Although over the next two decades various studies examined the relationships between rates of mortality and morbidity (Goodman *et al*, 1953) in the British population and a variety of risk factors including social class, air pollution and infection, it was not until 1957 that Stuart-Harris and co-workers published the landmark epidemiological study which clearly established the central role of cigarette smoking in the pathogenesis of lung disease. This and other papers reporting the adverse effects of smoking were summarised in 1962 in an important report on Smoking and Health from the Royal College of Physicians. The epidemiology of smoking related diseases has been examined at great length since then and recently in a review by Sherrill *et al*, 1990. The mortality rates for COPD increase with age and are higher in males than females. The risk factors for morbidity and mortality in COPD include smoking, occupational pollutant exposure and socio-economic status. COPD comprises three separate conditions, chronic bronchitis, emphysema and chronic asthma. In chronic asthma there is, along with other types of pathology, a loss of airway epithelium (Laitinen *et al*, 1985). Chronic bronchitis is defined as mucous hypersecretion with enlargement of trachea-bronchial submucosal glands and a disproportionate increase of mucous acini (Reid, 1968; Thurlbeck *et al*, 1975). Most patients with end-stage COPD also have severe emphysema (Thurlbeck, 1990).

Emphysema is a chronic disease involving progressive destruction of the distal airspace walls (Snider, 1992). The precise definition of this disease as affirmed by the National Heart, Lung and Blood Institute Workshop of 1985 (Snider *et al*, 1985) is that:

"Emphysema is a condition of the lung characterised by abnormal, permanent enlargement of air spaces distal to the terminal bronchioles accompanied by destruction of their walls and without obvious fibrosis".

The first clear anatomic description of emphysema was provided by Baillie in 1807, (Baillie, 1807), who suggested a breakdown of two or three contiguous cells into one, thereby forming a cell of a very large size. It was not until Laennec (Laennec, 1826, translated 1835) that the term emphysema was actually used. He recognised that fixation of the lung by inflation was essential to demonstrate the presence and extent of emphysema. His descriptions of the macroscopic pathology and clinical features of emphysema remained unaltered over the next 125 years.

It was almost half a century later in 1870 that the first recorded attempt to produce emphysema experimentally as made by Bayer occurred. He induced a pneumothorax in rabbits and noted emphysema developed at the margins of the contralateral lung. However, the indications are that he was simply observing the presence of the spontaneous disease consisting of subpleural bullae.

In the nineteenth and early twentieth centuries investigations were dominated by two theories of the pathogenesis of emphysema: the mechanical and the ischaemic. The mechanical theory, reported by Mendelssohn in 1845, is based on the principle that airflow limitation during expiration caused overdistention of distal airspaces with eventual mechanical destruction of alveolar walls and hence emphysema, particularly in the upper parts of the lung where distending forces were more pronounced and hence the upper lobe predominance of centralobular emphysema. The ischaemic theory was advanced to explain the disappearance of lung capillaries in emphysema and the development of alveolar fenestrae. Thrombosis of alveolar capillaries and the

obliteration of these vessels by increased alveolar pressure were invoked as the cause of impaired "nutrition" of the lung and tissue ischaemia (Waters, 1862), damaging the alveolar walls (Reviewed in Karlinsky & Snider, 1978).

Current theories of the pathogenesis of emphysema are based on work carried out over the last three decades. A landmark observation was made by Laurell & Eriksson in 1963 that a deficiency of the enzyme α -1-antitrypsin (α -1-proteinase inhibitor) was associated with the development of emphysema at the early age of 30 or 40. This coupled with the work of Gross *et al* (1965), who demonstrated that the induction of emphysema in healthy rats by intratracheal instillation of the crude form of proteolytic enzyme papain, led to the protease: antiprotease theory of the pathogenesis Emphysema. This hypotheses states that:

"an imbalance between proteases and antiproteases in the lower respiratory tract produces an excess of proteases, leading to proteolytic destruction of the connective tissue matrix which provides the architectural support for the alveolar walls" (Janoff, 1985). This hypothesis is based on a large body of work involving artificial, enzymatically induced animal models of emphysema and studies on emphysematous patients with and without α -1-antiproteinase deficiency. However the theory has been questioned recently (Morrison, 1987; Wewers *et al*, 1989).

The majority of patients with emphysema do not have a genetic deficiency of α -1-antiproteinase (Lieberman, 1969). The major factor in the aetiology of emphysema is cigarette smoking (Smoking and Health, 1984). It has been suggested that smoking may increase the elastase burden in the lungs by increasing the number of polymorphonuclear leukocytes in the distal airspace of smoker's lungs (Hunninghake &

Crystal, 1983). In addition, cigarette smoke has a high oxidative potential which could depress the function of elastase - inhibitors in the lungs (Dooley & Pryor, 1982), and also effect pulmonary elastin synthesis (Laurent *et al*, 1983).

It has also been suggested that an imbalance in the oxidant/antioxidant status (Janoff *et al*, 1983) of the lung is important in the development of emphysema. However, this has been a matter for much conjecture in the literature although recent data (Ogushi *et al*, 1991) has indicated an oxidant-antioxidant imbalance in the lower respiratory tract which could reduce the elastase inhibiting capacity in the lungs.

In its early stages emphysema is difficult to diagnose and it is not until marked tissue destruction has occurred that the condition presents clinically (Lamb, 1990). Attempts at early diagnosis in the past were based on establishing the most sensitive test of pulmonary function which could reliably predict the pathological changes either in resected or post-mortem lungs. Unfortunately, these structure-function studies have not shown that any test of lung function reliably predicts the presence of emphysema (Paré *et al*, 1982).

The first target for inhaled cigarette smoke is the airspace epithelium. Alveolar wall destruction including epithelial cells is a hallmark of emphysema. Thus the early injurious effects on the alveolar epithelium may be an important initial event in the pathogenesis of emphysema. It is for this reason that this thesis is focused on mechanisms of epithelial cell injury induced by cigarette smoke.

1.2. ALVEOLAR EPITHELIAL INJURY

One of the earliest signs of lung injury is an increase in airspace epithelial permeability. This has been observed as an early event in various

diseases, such as ARDS (Mason *et al*, 1985), acute asthma (Lemarchand *et al*, 1992), and also in otherwise healthy cigarette smokers (Jones *et al*, 1980). Exposure to hypoxia has also been shown to increase airspace permeability (Matalon & Egan, 1981; Nickerson *et al*, 1981) together with inhalation of the gaseous pollutants ozone (O₃) and Nitrogen Dioxide (NO₂) which are also components of cigarette smoke (Bhalla *et al*, 1986; Kehrl *et al*, 1987; Frampton *et al*, 1989). The increase in permeability which occurs with acute asthma or cigarette smoking is reversible on recovery from acute asthma (Lemarchand *et al*, 1992), or on cessation of smoking (Minty *et al*, 1981). In animal models, cigarette smoke has also been shown to increase airspace epithelial permeability in rat (Minty & Royston, 1985), rabbit (Witten *et al*, 1985) and guinea pig lungs (Simani *et al*, 1974; Burns *et al*, 1989). Electron microscopic examination in studies in the guinea pig showed damage to the type I epithelial cells at the level of the bronchiolar-alveolar junction (Burns *et al*, 1989). This supports work reported in 1980 by Reznik - Schuller who exposed Syrian golden hamster to cigarette smoke which produced cytoplasmic swelling and ruptured cell membranes of both type I epithelial cells and endothelial cells. Earlier work by the Vancouver group had suggested that there was disruption of the tight junctions of epithelial cells when animals were exposed to cigarette smoke (Boucher *et al*, 1980).

1.3. THE ALVEOLUS

The lower respiratory tract terminates in the alveoli. In general terms the alveolar wall consists of three tissue components: surface epithelium, connective tissue and the capillaries and their contents.

1.3.1. The Alveolar Epithelium

The epithelium forms a continuous alveolar lining to the alveoli consisting of two distinct cell types (Crapo *et al*, 1982; Haies *et al*, 1981). The type I pneumocyte is a large, extremely flattened cell with protuberant nuclei and highly attenuated cytoplasmic processes covering most of the alveolar surface (Kuhn, 1978) and thus constitutes the major part of the thin gaseous diffusion barrier.

The second cell is the type II pneumocyte, which is a large rounded cell with an extensively vacuolated cytoplasm and although present in large numbers only occupies approximately 3% of the alveolar surface area (Crapo *et al*, 1982). These cells actively transport solutes (Mason *et al*, 1982; Goodman *et al*, 1983), and have multilamellar bodies containing phospholipid which is discharged into the alveolar air space, contributing to the surfactant layer at the epithelial-air interface. (Dobbs *et al*, 1980; Whitsett *et al*, 1985). The type II cells have been shown to have the potential to proliferate and differentiate into type I pneumocytes in response to damage to alveolar walls (Evans *et al*, 1973; Adamson & Bowden, 1974; Adamson & Bowden, 1975). These cells also have the ability to produce and secrete components of the extracellular matrix (Sage *et al*, 1983; & Crouch *et al*, 1987).

The epithelial cells secrete and are subsequently situated on a basement membrane, comprised of extracellular matrix. This basement membrane defines and compartmentalises lung tissue, producing major structural support for the cells and exerts significant influence on cell growth, maturation and function (Crouch *et al*, 1991). The components of the basement membrane are collagen type IV, fibronectin, laminin and the proteoglycans. These will be described in the following section, which expands on the connective tissue of the lung with reference to the

alveolus.

1.3.2. Connective Tissue

The lung connective tissue is a complex mixture, containing collagen, noncollagenase glycoproteins, elastin and proteoglycans. Its structure and turnover is important in maintaining the integrity and function of the lungs. This tissue represents approximately 25% of the dry weight of the lungs (Hance & Crystal, 1975), with collagen being the most abundant and best-characterized component.

1.3.2.1. Collagen

Collagen is a glycoprotein, containing hydroxyproline and hydroxylysine residues. There are several different types of collagen which are present in the bronchi, arteries, veins and in the alveolar structure. At the alveolar level, collagen is present in the interstitium and in endothelial and epithelial basement membranes (Madri & Furthmayr, 1979). Types I and III account for at least 90% of the collagen in the adult lung parenchyma with two thirds of this thought to be type I collagen (Seyer *et al*, 1976). Type I collagen is present throughout the interstitium of the alveolar structure and is thought to be the major collagen type that comprises the banded fibres observed by electron microscopy (Huang *et al*, 1977). In culture, type I collagen has been used to promote cell attachment and growth (Reviewed in Klienman *et al*, 1981). Type IV collagen is localised to the epithelial and capillary basement membranes of the alveolus, representing approximately 5% of the parenchymal collagen (Rennard *et al*, 1980). It is different in structure to the interstitial collagens (I, II & III), being non-fibrillar and having pepsin-sensitive sites within the triple-helical portion of the molecule (Reviewed

Linsenmayer, 1981; Timpl, 1989). It also has a greater percentage of hydroxyproline and hydroxylysine residues than type I collagen. It forms a mesh with the basement membrane, presenting a barrier for diffusion of macromolecules or the movement of cells across the membrane (Timpl, 1989). Collagen is functionally important in promoting attachment and differentiation of epithelial and endothelial cells (Herbst *et al*, 1988), which are important events in repair and growth.

1.3.2.2. Noncollagenous Glycoproteins

Other major glycoproteins present in the lung and specifically the alveolar basement membrane are fibronectin and laminin.

1.3.2.2.1. Fibronectin

These are a class of compounds, which were proposed by Kuusela *et al*, (1976) to be collectively known as fibronectin. Fibronectins are present at the cell surface, in intercellular matrix, basement membranes and in the blood vessels (Reviewed, Hynes & Yamada, 1982). They can be defined in terms of three properties which they share: (1) they have two disulfide bonded subunits, with molecular weight near 220,000 daltons; (2) they bind to collagen, heparin and fibrin, and induce cell adhesion and spreading; and (3) they are antigenically cross-reactive irrespective of their source and species.

Fibronectin is associated with many cell types and is abundant at the interphase of epithelial cells and basement membranes. As previously mentioned, fibronectin has a number of functional properties including cell-cell, cell-matrix adhesion and cell spreading, which are important in growth and repair (Mosher, 1984; Campochiaro & Glaser, 1986).

1.3.2.2.2. Laminin

Laminin is the most abundant glycoprotein present in basement membranes (Review, Timpl & Dziadek 1986; Beck *et al*, 1990) and is present in the lung (Timpl *et al*, 1979). Like fibronectin it is a macromolecule with two disulfide bonds, but with a larger molecular weight of 800,000 daltons. It consists of one copy of each polypeptide chain designated A, B1 and B2, assembled into a cross-like structure (Engel *et al*, 1981) and exists as a noncovalent complex with the single polypeptide chain entactin/nidogen (Carlin *et al*, 1981; Paulsson *et al* 1987). Nidogen may mediate the bending of laminin to collagen IV although the nature of this protein and its potential binding are not fully established. Laminin binds to type IV collagen (Charonis *et al*, 1985) and heparin sulfate proteoglycan (Woodley *et al*, 1983) within the basement membrane.

Laminin influences many functions of cultured cells such as cell growth, survival, morphology, differentiation and motility (Kleinman *et al*, 1985; Herbst *et al*, 1988). It has also been suggested that laminin mediates the attachment of epithelial cells to type IV collagen.

Other components present in the connective tissue of the lungs are elastins and proteoglycans. These are described briefly below and their purpose within the lung connective tissue is explained although these components are not investigated further in this thesis.

1.3.2.3. Elastin

Elastin fibres are another important component of the interstitium of the lungs. These are composed of elastin microfibrils (Rosenbloom, 1987) and are responsible for the elastic recoil of lungs. They are found in close association with collagen fibres and proteoglycans (Rennard *et al*,

1980). There has been extensive investigation of the structure and function of elastin and its role in disease processes (Rosenbloom, 1987; Laurent *et al*, 1988).

1.3.2.4. Proteoglycans

The proteoglycans are macromolecules which make up the bulk of the ground substance. They are composed of a protein core with a number of covalently attached carbohydrate side chains and include chondroitin sulphate, heparin sulphate and hyaluronic acid. Heparin sulphate and possibly chondroitin sulphate chains are contained within the basement membranes of the lungs (Sannes, 1992; McCarthy & Couchman, 1990). As polyanionic sites, they serve as selective charge barriers and make the matrix impermeable to proteins under normal conditions (Farquhar, 1981).

Thus basement membrane components are important in maintaining the integrity and function of the lungs. There are differences in composition of the alveolar basement membrane associated with type I pneumocytes when compared with type II pneumocytes (Sannes, 1984; van Kuppevelt *et al*, 1984). This raises the question of relating distribution of components of basement membrane to their potential role in known functional characteristics (Sannes, 1991). The membrane also provides anchorage for the cell and so influences cell shape and function (Sugrue & Hay, 1981; Vlodavsky & Gospodarowicz, 1981; Rannels *et al*, 1987).

The ability of cells to attach to a matrix is crucial to growth and repair following injury. Attachment of cells to the matrix occurs at discrete sites on the cell surface via adhesion molecules (Buck & Horwitz, 1987).

1.4. ADHESION MOLECULES

The cell surface adhesion molecules can be described in three distinct groups: (1) those present on the luminal surface of the cell during inflammation that function as cell-cell adhesion molecules, important for example in white blood cell-endothelial cell adhesion; (2) those present at the cell-cell borders of adjacent cells that function to maintain cell-cell adhesion; and (3) those present on the basement membrane side of the cell that function as cell-substratum adhesion receptors.

It is the receptors specifically involved in basement membrane attachment that are of interest to this thesis. The best characterised of these molecules are the integrins.

1.4.1. Integrins

Integrins are a family of heterodimeric glycoproteins that mediate cell-to-cell and cell-to-extracellular matrix interactions (McDonald, 1989, Hemler, 1990; Ruoslahti, 1991; Hynes, 1992). Each member of this family is composed of a single α subunit and a single β subunit that are noncovalently linked. Originally the integrins were divided into 3 subfamilies, each with a common β subunit capable of associating with a specific group of α subunits (Hynes, 1987). Since then other β subunits have been isolated (Cheresh *et al*, 1989, Sheppard *et al*, 1990; Moyle *et al*, 1991) and with the presence of certain promiscuous α subunits, which are α_4 , α_6 and α_v , the classification of the subfamilies has become less well defined.

All integrins appear to interact with ligands in a cation-dependent fashion (Hynes, 1987; Gailit & Ruoslahti, 1988). Many but not all integrins appear to bind to ligands at sites containing the tripeptide arginine-glycine-aspartic acid (RGD) (Pytela *et al*, 1986; Gailit & Ruoslahti, 1988).

These integrins are an important factor in maintaining the integrity of the alveoli but cell surface receptors are vulnerable to proteolytic cleavage (Spertini *et al*, 1991) and this cleavage could lead to disruption of the epithelium.

Both of the subunits of the integrins are transmembrane glycoproteins containing extensive disulfide bonds (Calvete *et al*, 1991). The cytoplasmic domains are generally relatively short and are believed to interact with the cytoskeletal proteins (Reviewed, Burridge *et al*, 1988). The interdependence of these two facets of the cell are critical to many cell functions such as cell motility, which with respect to the epithelial cell is important in the repair process. The relationship between the integrins and the cytoskeleton is also likely to be critical to the maintenance of the barrier function of the epithelium.

1.5. CYTOSKELETON

The cytoskeleton is responsible for both cell shape and internal organisation. Two of the most important components of the cytoskeleton are the microtubules and the microfilament, both of which are active components with the ability to rapidly assemble and disassemble (Albert *et al*, 1989).

1.5.1. Microtubules

The microtubules are hollow cylinders composed of tubulin which during mitosis construct the mitotic spindle. The main function of microtubules is to contribute to the maintenance of cell shape and to permit intra- and extracellular movement (Hyams & Stebbings, 1979). Tubulin is a globular polypeptide of 50,000 daltons and is a dimer consisting of an α and β subunit in a dynamic state of assembly and disassembly.

Microtubule-associated proteins have as one of their functions responsibility for the movement of internal organelles.

1.5.2. Microfilament

The microfilaments consist of actin, which is present in two forms in the cell (1) filamentous actin, or F-actin and (2) G-actin which is the globular form (43,000 daltons) and is the most abundant protein in cells. The actin filament is a polarised structure which undergoes "Treadmilling", an active process with the addition of G-actin at one end and the loss of actin-monomers at the other. It provides mechanical support in maintaining cellular structure, and is part of the contractile system responsible for locomotion (Reviewed Korn, 1982). The microfilaments interact with other proteins in the cytoplasm, which form cross-links between actin-filaments and connections with the plasma membrane amongst other functions (Carraway & Carraway, 1989).

1.6. CELL SIGNALLING

For a cell to respond to an external signal or event, it requires a complex system of internal signalling to differentiate between the myriad of signals to which it is exposed. The transmission of these external signals into functional changes in the cell is achieved by the so called signal transduction pathways. This thesis focuses on three key elements of this signal transduction, (1) the protein kinases, (2) calcium, (3) the regulatory G-proteins.

1.6.1. Protein Kinases

The existence of protein kinases with a regulatory function was first suggested by the observations of Fischer & Krebs (1955) and Rall *et al*,

(1957). They noted the presence in animal cells of both an active phosphorylated and an inactive dephosphorylated form of the enzyme glycogen phosphorylase. Protein kinases catalyse the transfer of a phosphate moiety from MgATP^2 to a serine, threonine or tyrosine residue in the polypeptic chain of a target protein. Phosphorylation of the amino-acid residue alters the conformation of the target protein and causes either an activation or an inhibition of the function of the target protein.

1.6.1.1. Cyclic-AMP Dependent Protein Kinase

The first protein kinase enzyme to be discovered, cyclic AMP-dependent protein kinase, was isolated in the 1960's (Huijing & Larner, 1966; Walsh *et al*, 1968). There are two forms of cyclic AMP-dependent protein kinase present in animal cells and in the absence of an activator these exist as a dimer composed of two catalytic and two regulatory subunits. The target proteins phosphorylated by cyclic AMP-dependent protein kinases in intact cells include a large number of regulatory enzymes, enzymes involved in muscle contraction, and RNA polymerase.

1.6.1.2. Protein Kinase C

Protein kinase C (PKC) was first described but not named by Nishizuka and his colleagues in 1977, it was found to be activated by proteolytic cleavage (Inoue *et al*, 1977). Here workers subsequently showed that the enzyme requires phospholipid, Ca^{2+} and diacylglycerol for maximum activity. Nishizuka proposed that the major physiological activator of PKC was the diacylglycerol which is formed in the plasma membrane by the hydrolysis of phosphoinositides and this has also been found to be dependent on Ca^{2+} . PKC is actually comprised of a family of eight or more closely related isozymes (Parker *et al*, 1989). It is presumed that

the different species of PKC perform different cellular functions although these functional differences have not yet been clearly defined. The major target proteins are plasma-membrane receptors, other plasma-membrane proteins, proteins of the cytoskeleton, contractile proteins, regulatory enzymes in metabolic pathways and sequence-specific DNA-binding proteins involved in the control of gene expression and cell proliferation. Many of the target proteins for PKC are also substrates for cyclic AMP-dependent protein kinases.

1.6.2.Calcium

Calcium plays an important role in most cells as an intracellular messenger. Ringer reported in 1883 the requirement of extracellular Ca^{2+} for contraction of cardiac muscle. Subsequent experiments by many investigators established the role of Ca^{2+} in heart and skeletal muscle, the first of these being Heilbrunn & Wiercinski (Heilbrunn, 1948; Heilbrunn & Wiercinski, 1947). The early 1970's led to the accumulation of evidence that changes in $[\text{Ca}^{2+}]_i$ were required for coupling between extracellular stimuli and cellular responses in non-muscle cells. By 1981 it was possible to measure $[\text{Ca}^{2+}]_i$ in the cytoplasmic space (Tsien, 1981) and so it was possible to measure changes in $[\text{Ca}^{2+}]_i$ induced by agonists.

There is a large gradient of Ca^{2+} between the extracellular fluid and the cytoplasmic space. The concentration of intracellular calcium ($[\text{Ca}^{2+}]_i$) is about 0.1 nM where the concentration of free Ca^{2+} in the extracellular environment is greater than $1\mu\text{M}$. An increase in the concentration of $[\text{Ca}^{2+}]_i$ acts as an intracellular second messenger for the action of many agonists. Ca^{2+} binds to sites on target proteins leading to alterations in enzyme activity and protein function. One such protein is Calmodulin

which itself interacts with a wide variety of proteins. Amongst these are calmodulin-dependent protein kinases, Ca^{2+} transporters and components of the cytoskeleton. There are also another group of Ca^{2+} modulated proteins which include protein kinase C, Ca^{2+} activated ion channels and phospholipases A and D. It is important to remember that signals from the external environment can and do activate more than one pathway of intracellular communication. In many cases the signal pathway connecting the signal received by the receptor and the generation of the second messenger is through the Guanine nucleotide regulatory proteins.

1.6.3. Guanine Nucleotide Regulatory Proteins

These GTP-binding regulatory proteins (G proteins) play a central role in transmembrane signalling events which are responsible for coupling between receptors and effector enzymes or ion channels (Reviewed Gilman, 1987; Birnbaumer & Brown, 1990). The role of GTP was first demonstrated by Rodbell and colleagues (1971) and evidence then accumulated suggesting the interaction of GTP with a neighbouring protein located in the plasma membrane (G protein). Much of the early work was based on the activation of adenylate cyclase. Direct evidence of a role for G-proteins in this activation was obtained by Ross & Gilman (1977). With the subsequent development of methods to purify G-proteins (Schleifer *et al*, 1980), more in depth investigation of their mode of action was possible.

It is now widely recognised that the G-protein family participate in regulation of cyclic GMP-specific phosphodiesterase (Wheeler & Bitensky, 1977), phospholipase C (Reviewed Harden, 1990), phospholipase A_2 (Burch *et al*, 1986) and stimulation of K^+ and Ca^{2+} channels (Yatani *et al*, 1987; Jones *et al*, 1988).

The cell signalling allows the cells to react to an outside stimulus. The alveolus is an area that is exposed to inhaled toxins, whether natural or manmade. One such manmade toxin is cigarette smoke.

1.7. CIGARETTE SMOKE AND ITS` CONDENSATES

An average cigarette smoker in the UK consumes 2,120 cigarettes a year or approximately 6 cigarettes per day (Mossman & Eastman, 1987). Cigarettes contain more than 6,000 different components. Each puff of cigarette smoke contains at least 10^{16} oxidant molecules of which 10^{14} are oxygen radicals (Pryor *et al*, 1981), so that the gas phase of tobacco smoke is a rich source of oxidants (Pryor *et al*, 1983). Many of the other components of cigarette smoke are also toxic, such as Cadmium, Nickel, Benzo(a)pyrene, Nicotine, Formaldehyde, Nitrogen oxides, Hydrogen Cyanide, Acrolein, and Vinyl Chloride (Stedman, 1968) to name but a few. These compounds can be carried in either the vapour or the particulate phase of cigarette smoke into the lungs. Cigarette smoke can be used experimentally as a whole, containing both the vapour phase and the particles or purely as the vapour phase (Kilburn & McKenzie, 1975; Bridges, 1985; Minty & Royston, 1985). The vapour phase can be obtained by passing the whole smoke through a glassfibre Cambridge filter, which is capable of removing 99.9% of all particles $\geq 0.1 \mu\text{m}$ in diameter (Guerin, 1980). The exact nature and composition of the inhaled cigarette smoke which reaches the alveolus is unknown. However a percentage of the smoke will dissolve in the epithelial lining fluid (ELF) in the moist atmosphere of the airspaces. Many different cigarette smoke extracts have been artificially produced in an attempt to mimic the components occurring *in vivo* in the ELF of smoker's lungs. The effects of the neutral, acidic and basic component of the cigarette

smoke extract or condensate on elastin or cell cultures have been examined (Laurent *et al*, 1983; Miyashita *et al*, 1990). Extracts of whole or vapour phase smoke have also been produced by passing the cigarette smoke through a variety of buffers, such as Tris (Wyss *et al*, 1984; Rickard *et al*, 1992), phosphate buffer (Pryor & Dooley, 1985; Holden *et al*, 1989), water (Bridges, 1985; Richards, 1991), or the widely used organic solvent dimethylsulfoxide (Janoff & Carp, 1977; Holden *et al*, 1989). Their effects on different components of the lung, α -1PI or elastin, or cell types, i.e., bronchial epithelial cells, Clara cells in the lung have been studied. It has been suggested that although many of the reactive oxidant intermediates are short lived and travel a relatively short distance before being quenched, cigarette smoke condensate formed in the ELF continues to produce oxidants (Pryor 1986).

Experimentally, exposure of specific pathogen-free rats (S.P.F.) to cigarette smoke for 2-6 weeks can induce similar histological changes in the large airways to those seen in the lungs of chronic bronchitics (Jones *et al*, 1972; Jeffery & Reid, 1981). Increases in epithelial thickness, secretory cell number and cell division have all been reported (Rogers & Jeffrey, 1986). Serous cell metaplasia occurs resulting in a greater proportion of mucous cells and the epithelium thickens with cell hypertrophy. This increase was proportional to the duration of exposure to cigarette smoke (Jeffery & Reid, 1981).

Although cigarette smoking is thought to be the major aetiological factor in the development of emphysema, the production of an animal model of emphysema using exposure to cigarette smoke alone has been relatively unsuccessful. Using a dog model of chronic cigarette smoke exposure Park and colleagues (1977) failed to produce a decrease in alveolar surface area which is a hallmark of emphysema. Other studies of smoke

exposure in the hamster showed the development of alveolar and peribronchiolar inflammation (Dontenwill *et al*, 1973; Hoidal & Niewoehner, 1982), but again the animals did not develop emphysema. Investigations in the rat also failed to find convincing characteristic changes in the lung. Heckman & Dalbey (1982) noted a slight increase in mean linear intercept, while Huber and co-workers (1981) found a decreased alveolar surface area in the rat. Detailed pulmonary function tests and analyses of the lung structure however were not carried out. Wright and Churg (1990) reported a guinea pig model of smoke exposure which resulted in progressive lung destruction, which they termed "emphysema", and showed characteristic changes in pulmonary function tests. The caveat to these experiments was that there was no difference in the extent of tissue destruction between upper and lower lobes. In humans, smoke-associated emphysema is usually worse in the upper lobes. Moreover there was no marked centrilobular predominance in the animal lungs which is the characteristic distribution of alveolar destruction in human lungs.

Since the air spaces of the lung are in direct contact with the environment, they are therefore exposed to many noxious substances such as oxidants and must therefore have mechanisms to protect against injury.

1.8. DEFENCE SYSTEMS

The lung has both intra and extra cellular mechanisms, which can protect against the acute effects to an inhaled insult. They may also adapt to a chronic exposure. There are both anti-protease and anti-oxidant protective mechanisms in the lungs. Direct exposure to cigarette smoke imposes an enormous oxidative burden on lung cells. It is therefore the anti-oxidant protective mechanisms which will be the main focus of this

thesis.

1.8.1. Free Radicals

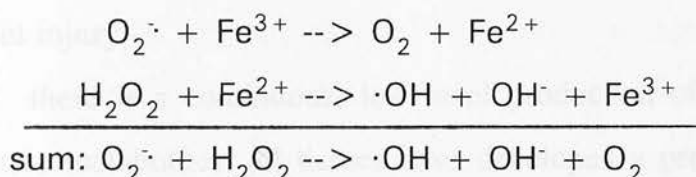
A free radical is any species which has one or more unpaired electrons. This is a broad definition that includes the hydrogen atom, most transition metals and oxygen (O_2) itself, with two unpaired electrons. A large percentage of the O_2 consumed by mammalian cells is reduced to form water (H_2O). However, oxygen can also be partially reduced to form the reactive oxygen intermediates or metabolites, which include the superoxide anion, hydrogen peroxide, the hydroxyl radical and hypohalous acids. It was the formation of these reactive oxygen intermediates that was proposed to cause the injury produced by exposure to high concentrations of oxygen (Reviewed in Halliwell & Gutteridge, 1984).

1.8.1.1. The Reactive Oxygen Intermediates

The superoxide anion ($O_2^{\cdot-}$), is known to be formed *in vivo* (Fridovich, 1989; Halliwell & Gutteridge, 1989). Some of this $O_2^{\cdot-}$ production is accidental, e.g. by leakage onto O_2 of electrons from the electron transport chains of the mitochondria and the endoplasmic reticulum. Deliberate $O_2^{\cdot-}$ release occurs from phagocytic cells (Curnutte & Babior, 1987), activated in response to foreign bodies. The phagocytic leukocytes in the lungs contain NADPH oxidase and it is the oxidases in cells that are known to produce $O_2^{\cdot-}$ directly or indirectly. In aqueous solution $O_2^{\cdot-}$ is converted to hydrogen peroxide (H_2O_2) and O_2 by spontaneous dismutation, which is enhanced in the presence of the enzyme superoxide dismutase (SOD). This can be summarised as follows:



Both $\text{O}_2^{\cdot-}$ and H_2O_2 can directly damage cellular components (Fridovich, 1989), but in general their reactivity is limited (Fee & Valentine, 1977). Much of the damage done by $\text{O}_2^{\cdot-}$ and H_2O_2 in vivo is thought to be due to their conversion into highly reactive oxidants (Sutton & Winterbourn, 1989). One of these oxidants is the hydroxyl radical ($\cdot\text{OH}$) (McCord & Day, 1978). Formation of $\cdot\text{OH}$ from $\text{O}_2^{\cdot-}$ requires traces of catalytic transition metal ions, of which iron seems likely to be the most important in vivo (Halliwell & Gutteridge, 1988), although copper ions might also play a role (Sutton & Winterbourn, 1989). The classic pathway for the $\cdot\text{OH}$ formation involving iron is the Haber-Weiss reaction (1934):



Formation of $\cdot\text{OH}$ in vivo is potentially very injurious. The radical reacts with DNA in vitro to cause strand breakage (Bawn & Fridovich 1981; Schraufstatter *et al*, 1986). The consequences of this in vivo are mutagenic and carcinogenic responses (Ames, 1983). The hydroxyl radical is also known to damage proteins producing cross-linking and aggregation (Freeman & Crapo, 1982), as well as oxidising the SH groups present at the active site of essential enzymes (Cochrane *et al*, 1983). Lipid peroxidation, is also thought to occur in the presence of $\cdot\text{OH}$ (Aust & Svingen, 1982). This leads to loss of the functional integrity of cell membrane, with changes in fluidity and permeability

which could eventually lead to cell death.

If $\cdot\text{OH}$ formation occurs in the proximity of myeloperoxidase and Cl then the potent cytotoxic oxidant hypochlorous acid (HOCl) results. This can also react with bases within DNA and degrade amino acids and proteins (Grisham & McCord 1986).

It is also worth noting that $\text{O}_2^{\cdot-}$ or H_2O_2 can react with the antioxidant reduced glutathione (GSH) to produce reactive sulphur-containing radicals that themselves may be more injurious than $\text{O}_2^{\cdot-}$ and H_2O_2 (Wefers & Sies, 1983; Schoneich *et al*, 1989). The $\cdot\text{OH}$ radical can also react with thiols to produce thiyl radicals ($\text{RS}\cdot$) and can then further react with oxygen to give reactive oxysulphur radicals such as $\text{RSO}\cdot$ and $\text{RSO}_2\cdot$ (thiyl peroxy). These radicals appear to be capable of inactivating antiproteinases (Aruoma *et al*, 1989). Thus it is possible by a series of reactions that an antioxidant can itself become an oxidant giving rise to oxidant injury.

Since there is a continuous, low level production of cytotoxic reactive oxidative metabolites, all tissues have developed a protective mechanism against their injuries.

1.8.2. Antioxidants

A major constituent of the protective mechanisms in the lungs are the antioxidants. An antioxidant is a substance that, when present in low concentrations compared to those of an oxidisable substrate, significantly delays or prevents oxidation of that substrate (Halliwell, 1990). Almost every component of lung cells including proteins, lipids, carbohydrates and DNA, can be termed an "Oxidisable substrate".

This thesis is focused on the protective properties of the following major antioxidants in the lung: the specific antioxidants superoxide dismutase

(SOD) and catalase and the non-specific antioxidant reduced glutathione (GSH).

1.8.2.1. $O_2^{\cdot-}$ Inhibition:- Superoxide Dismutase

Superoxide dismutase is an enzyme which ameliorates the toxicity of $O_2^{\cdot-}$ by the process of dismutation. The notion that this was an important defence mechanism was first put forward in 1971 by McCord *et al*, in a study of the content of SOD in aerobes, microaerophiles and obligate anaerobes. Upon exposure of *E. coli* and *Streptococcus faecalis* to oxygen there was increased biosynthesis of SOD, which correlates with an increased tolerance to the effects of hyperoxia. (Gregory & Fridovich, 1973). Through evolution this enzyme developed into two distinct families. The first family consists of the manganese-containing superoxide dismutases (MnSODs), found in prokaryotes and in the matrix of mitochondria, and the related iron-containing superoxide dismutases (FeSODs), which are also found in prokaryotes and a few plant families. The second is in the unrelated copper and zinc superoxide dismutases (Cu,ZnSODs) which occur primarily in the cytosol of eukaryotic cells and in chloroplasts (Fridovich, 1986).

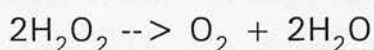
The prevalent enzyme in humans is Cu,ZnSOD, which is a stable dimeric protein of 32,000 daltons (Fridovich, 1983). Copper is essential for the enzyme's catalytic activity and zinc imparts stability to the protein structure (Fridovich, 1975). The enzyme is present intracellularly with very little present in the extracellular fluids.

The therapeutic potential of SOD is limited by its short half-life (Beckman *et al*, 1988), its sensitivity to proteases and its inability to penetrate cell membranes. This inability to confer protection against the lethal effects of 100% O_2 in rats was demonstrated by Crapo and colleagues (1977). It

is only with the covalent attachment of SOD to the inert linear polymer, monomethoxy-polyethylene glycol (PEG) or by its encapsulation in liposomes that this enzyme demonstrates its protective potential experimentally (Turrens *et al*, 1984; Beckman *et al*, 1988; Walther *et al*, 1991).

1.8.2.2. H₂O₂ Inhibition:- Catalase

Catalase is a 240,000 MW tetrameric haemoprotein that undergoes alternate divalent oxidation and reduction at its active site in the presence of H₂O₂ (Deisseroth & Dounce, 1970; Chance *et al*, 1979) and catalyses the dismutation reaction:



It is located primarily in peroxisomes, being highly compartmentalised in mammalian cells (Chance *et al*, 1979; Fridovich, 1983). Catalase is also present in large quantities in the ELF and is presumed to be released by dying epithelial cells trapped in the slowly exchanging fluid on the alveolar surface (Hubbard *et al*, 1987).

As with SOD, lung catalase activity can be enhanced. This has been achieved experimentally in animals by pre-exposure to endotoxin before the insult of 100% O₂, conditions which are associated with enhanced survival following exposure to hyperoxia (Frank *et al*, 1980). Both covalent attachment of catalase to PEG or its encapsulation in liposomes, as with SOD, also reduces the vulnerability of tissues to oxidative stress. (Turrens *et al*, 1984; Padmanbhan *et al*, 1985; Buckley *et al*, 1987).

1.8.2.3. ·OH Inhibition:- Desferrioxamine

While there are antioxidants present in the lung which can scavenge oxidants such as $O_2^{\cdot -}$ and H_2O_2 , another protective mechanism is thought to be present for $\cdot OH$. The mechanism by which damage caused by $\cdot OH$ can be ameliorated in vivo is binding of the transition metal ions required for its formation from $O_2^{\cdot -}$ and H_2O_2 (Halliwell & Gutteridge, 1989). This will alter the redox potential and/or accessibility of the metal ion so that it cannot participate in $\cdot OH$ formation. It has been argued that iron and copper ion transport and storage proteins have evolved so as to safely sequester the metal ions that they transport or store in forms that are incapable of stimulating free radical reactions (Gutteridge & Stocks, 1981; Halliwell & Gutteridge, 1986). A large percentage of the body's iron is bound to haemoglobin (Aisen & Listowsky, 1988), while the majority of the remaining extracellular iron is bound as the ferric ion to transferrin and lactoferrin, which are iron-binding glycoproteins (Gutteridge, *et al*, 1981; Gutteridge, 1986). These are also scavengers of free radicals (Goldstein *et al*, 1982). The remainder of the body's iron is present in the intracellular storage proteins, ferritin and haemosiderin. These metal ions can be released from stores for normal physiological use, but this also makes iron available for free radical reactions (Halliwell & Gutteridge, 1986).

Administration of the iron chelator, desferrioxamine, is likely to protect against damage mediated by $\cdot OH$ by binding iron (Halliwell 1989). This has been observed experimentally, in vitro, by its ability to protect cultured endothelial cells against injury from stimulated neutrophils (Varani *et al*, 1985) and rat alveolar type II cells against cell lysis induced by the toxin paraquat (van Der Wal *et al*, 1992). Lung injury induced in vivo, in the hamster (Chandler *et al*, 1985) and in the rat (van Asbeck *et*

al, 1989) can also be inhibited by the administration of desferrioxamine. Unfortunately, desferrioxamine is toxic to mammalian systems since it inhibits protein synthesis.

1.8.2.4. Glutathione

Glutathione is a tri-peptide which is synthesised intracellularly by a two-step process. The first and rate-limiting step is the ATP-dependent reaction of glutamate and cysteine to form γ -glutamylcysteine, catalysed by γ -glutamylcysteine synthetase. Glycine is then added in a second ATP-dependent reaction catalysed by glutathione synthetase (Snoke & Block, 1954). In cells *in vivo*, regulation of the intracellular level of glutathione is achieved by a combination of feedback inhibition of γ -glutamylcysteine synthetase, by the end product glutathione and by availability of the amino acid substrate required for the reaction (Richman & Meister, 1975; Deneke & Fanburg, 1989). In the presence of the enzyme glutathione peroxidase, GSH is oxidised to form glutathione disulfide (GSSG). The disulfide can be reduced back to GSH in a reaction catalysed by the enzyme glutathione reductase.

Glutathione is the most abundant intracellular non-protein thiol compound. It has a number of important cellular functions, mainly related to the thiol group of its cysteine residue (Meister & Anderson, 1983). In particular, it has an essential role as a co-substrate for the enzyme glutathione peroxidase, which appears to act to detoxify lipid peroxidases in the cell, thereby reducing the potential damaging effects of oxygen free radical production.

Many cells appear to have an active transport system to remove oxidised glutathione from the cell (Chance *et al*, 1979). An increased release of oxidised glutathione from cells may therefore reflect an insult to the cell

from oxidising free radical species.

Intracellular levels of glutathione differ dramatically between cells of differing origins (Kosower & Kosower, 1978; Allalunis-Turner *et al*, 1988). Even in the same cell type, glutathione levels change as a function of cell culture conditions and the stage of cell growth (Harris & Patt, 1969; O'Hara & Terasima, 1969; Harris & Teng, 1973; Post *et al*, 1983; Meredith, 1986). The highest levels of glutathione are observed during the early portion of the exponential phase of cell growth and the lowest levels are found in the plateau phase of the A549 epithelial cell line (Post *et al*, 1983). A similar phenomenon has been reported in other cells (O'Hara & Terasima, 1969; Harris & Patt, 1969; Allalunis-Turner *et al*, 1988).

ELF of the lower respiratory tract contains large amounts of glutathione. The total glutathione concentration of normal ELF is 140-fold higher than that of the plasma from the same individual and 96% of the glutathione is in the reduced form. Compared with non-smokers, cigarette smokers have >80% higher levels of total glutathione in ELF, 98% of which is in the reduced form (Cantin *et al*, 1987). The average concentration of GSH in ELF of non-smokers has been calculated to be approximately 400 μ M whilst in smokers this value was nearly 800 μ M. This increase in the level of GSH in ELF from smokers is also reflected in the higher levels of intracellular GSH in smoker's erythrocytes compared to non-smokers (Toth *et al*, 1986). However, a 50% reduction in the GSH concentration in isolated rat and rabbit lungs (Joshi *et al*, 1988) and up to 70% in a rat model have been observed following acute cigarette smoke exposure (Moldeus *et al*, 1985).

Since the direct effect of cigarette smoking is the focus of this thesis, as cigarette smoke is predominantly an oxidant toxin, the role of the

proteases/antiproteases, discussed in Section 1.1., is examined only briefly. The general purpose of this thesis was: (1) to develop an in vitro model of alveolar epithelial cell injury and repair in response to cigarette smoke and cigarette smoke condensates; (2) to examine the protective properties of certain antioxidants and antiproteinases within the systems; and (3) to discover the mechanisms of cigarette smoke induced cell damage and how this effects epithelial cell function. This was achieved using 3 different types of epithelial cells in functional assay systems.

CHAPTER 2

MATERIALS AND METHODS

CHAPTER 2

MATERIALS AND METHODS

2.1. MATERIALS

The following chemicals were supplied by:

BDH, Poole, Dorset, U.K.

Acetic acid, Anala, Dorset, U.K.

Butyleneglycoltriacetic acid, Formula Glycol, Hydrogen peroxide,

Sodium chloride,

MATERIALS AND METHODS

Calbiochem, Nottingham, U.K.

Calphostin C, Forskolin, 1-(5- α -cholestano-3 β -ol-20-one)-1-methylpiperazine,

CHCl₃ (H 7), N-[2-(methyl amino)-ethyl]-1H-benzotriazin-4-ylmethylcarbamate,

CHCl₃ (H 8)

Citro, Paisley, U.K.

Cowing serum albumin fraction V, Deftex, phosphate buffered saline

Eagle minimal essential medium, fetal calf serum, L-Glutamine, Hanks

buffered salt solution (with/without Ca²⁺, Mg²⁺), DMEM medium,

Sodium bicarbonate, Tissue Culture, Vervac.

Scotlec, Kidlington, Oxford, U.K.

Monoclonal antibodies to the integrin CD25 (clone B-DU), CD44 (clone

ANK), CD45 (clone H22L), CD46 (clone SAM), CD47 (clone

CD-113).

CHAPTER 2

MATERIALS AND METHODS

2.1. MATERIALS

The following chemicals were supplied by:-

BDH, Poole, Dorset, U.K.

Acetic acid, Azide, Dimethyl sulfoxide, Ethylenediaminetetraacetic acid, Ethyleneglycoltetraacetic acid, Formalin, Glyceel, Hydrogen peroxide, Sodium chloride.

Calbiochem, Nottingham, U.K.

Calphostin C, Forskolin, 1-(5-isoquendolinesulfonyl)-2-methylpiperazine, diHCl (H-7), N-[2-(methyl-amino)-ethyl]5-isoquinoline-sulfonamide, diHCl (H-8).

Gibco, Paisley, U.K.

Bovine serum albumin fraction V, Dulbecco phosphate buffered saline, Eagles minimal essential medium, Fetal calf serum, L-Glutamine, Hanks buffered salt solution (with/without Ca^{2+} , Mg^{2+}), LHC-9 medium, Sodium bicarbonate, Trypsin-EDTA, Versene.

Serotec, Kidlington, Oxford, U.K.

Monoclonal antibodies to the integrins CD29 (clone B-D15), CDw49b (clone AK7), CDw49d (clone HP2/L), CDw49e (clone SAM1), CDw49f (clone GO-H3).

Sigma, Poole, Dorset, U.K.

Arginine-glycine-aspartic acid, Arginine-glycine-glutamine-serine, 2-Amino-2-methyl-1-propanol buffer, Alpha-1-proteinase inhibitor, Bovine serum albumin, L-Buthionine-SR-sulfoxamine, Catalase, Collagen I, Collagen IV, Colchicine, Cysteine, Cytochalasin B, Cytochalasin D, Deoxyribonuclease 1 (crude form, DN-25), Desferrioxamine, Dibutyl cAMP, Fast red, Ferricytochrome C, Fibronectin, N-Formyl-met-leu-phe, Horse radish peroxidase (type II, salt free), Glutathione (Reduced form), Laminin, Lipopolysaccharide (L-2630), Lysophosphatidyl choline, Naphthol AS phosphate, Pertussis toxin, Phalloidin-FITC labelled, Phenol red, Phorbol 12-13 dibutyrate, Phorbol 12-myristate 13-acetate, Phorbol 12-myristate 13-acetate 4-O-Methyl ether, Sphingosine, Sodium hydroxide, Soya bean trypsin inhibitor, Superoxide dismutase.

The suppliers of the miscellaneous products were as follows:-

Amersham International, Amersham, Bucks, U.K.:- Sodium chromate, Tritiated thymidine.

Bantin & Kingman Universal, Hull, U.K.:- Male Wistar rats.

Baxter Healthcare, Norfolk, U.K.:- Diff-Quik, Sterile water, Sterile saline.

Bayer Diagnostics, Basingstoke, Hampshire, U.K.:- OCT.

Britannia Pharmaceuticals Ltd., Redhill, Surrey, U.K.:- Benzylpenicillin.

DAKO, High Wycombe, U.K.:- Rabbit-anti-mouse biotin, Rabbit-anti-rat biotin.

Evans, Horsham, U.K.:- Streptomycin.

Genzyme, West Malling, Kent, U.K.:- Tumour necrosis factor.

Koch-Light Lab. Ltd., Colnbrook, Bucks, U.K.:- Triton-X-100.

Molecular Probes, Eugene, Oregon, U.S.A.: Rhodamine-conjugated Phalloidin.

Pharmacia LKB, Uppsala, Sweden: Percoll.

Rhone-Merieux, Harlow, U.K.: Nembutal.

Roussel Lab Ltd., Uxbridge, U.K.: Gentamycin M.

Vector Labs, Burlingame, CA, U.S.A.: Vectastain ABC Elite kit.

Gifts:-

Dr. I. Dransfield, University of Edinburgh:- monoclonal antibodies against α_v and $\alpha_v\beta_3$, and 52 μ an irrelevant integrin.

Dr. M. Bridgeman, University of Edinburgh:- NAC.

2.2. METHODS

2.2.1. EPITHELIAL CELLS

Three different types of epithelial cells were used in the following studies: (a) the alveolar epithelial cell line, A549; (b) the bronchial epithelial cell line, BEA-2B; (c) primary cultured rat alveolar type II cells. In addition an attempt was also made to isolate human type II cells.

The cell of primary interest was the A549 epithelial cell as the main interest of this thesis was the alveolar epithelium. This cell line was used because it was derived from a human alveolar epithelial cell and was inexpensive and easy to grow and maintain. In an ideal situation human primary culture type II cells would be the best to use but these are difficult and time consuming to isolate and maintain. This will be discussed in greater depth in Chapter 7.

However, primary culture rat type II cells are used in selected experiments for comparison. The bronchial epithelial cell line, BEA-2B

was also studied in a limited number of experiments for comparison also. This was used as it was an immortalised non tumour-derived lung epithelial cell line.

(a) A549 Epithelial Cells

The alveolar epithelial cell line, A549, was established in January, 1972 and first reported by Giard *et al*, (1973). The cell line was derived from a tumour in the right lower lobe of a 58-year-old Caucasian male, which histologically was an alveolar cell carcinoma with cuboidal epithelial tumour cells lining the alveoli, and mucinous material filling the acinar-like spaces.

It was observed that early (12) and late passage (112) cells contained multilamellar bodies and synthesised relatively large quantities of disaturated lecithin. This synthesis is an indication that the cells have retained differentiated functions. This was a typical transferred cell line with the *in vivo* growth criteria of :- colony formation in soft agar, growth on top of a fibroblast monolayer and when injected into immunosuppressed mice progressively growing tumours were formed.

The Lieber group (1976) confirmed lecithin synthesis in A549 cells; obtaining similar data from whole lung tissue and isolated rabbit type II pneumocytes. The pattern of phospholipid synthesis in A549 cells was as expected for type II pneumocytes which synthesised pulmonary surfactant. The alveolar type II epithelial cell line, A549, was maintained in continuous culture, in 75ml flasks incubated at 37°C, 5% CO₂ in Eagles Minimal Essential Medium (MEM), which contained penicillin (100 µg.ml⁻¹), streptomycin (50µg.ml⁻¹), L-Glutamine (20mM), Sodium bicarbonate, and 10% Fetal Calf Serum (FCS).

When required for the assays, confluent monolayers of A549 epithelial

cells were washed twice with Dulbecco Phosphate buffered saline (PBS). Thereafter, Trypsin-EDA solution was added to detach the cells. The cells were then washed with MEM, containing 10% FCS at 200g for 10 minutes, to neutralise the trypsin, and resuspended in MEM with 10% FCS. This cell suspension was then used in all the assays described in the Methods section.

(b) BEA-2B Epithelial Cells

The BEA-2B epithelial cell line was first reported by Reddel and colleagues (1988). The cell line was derived from normal human bronchial epithelial cells cultured from explants of autopsy specimens from non-cancerous individuals. These cells were transformed using the adenovirus 12-SV40 hybrid virus. On examination there was no evidence of viral persistence in the epithelial cells. Electron microscopy of these cells revealed desmosomes and tight junctions, with indirect immunofluorescence showing evidence of the presence of keratin. When the cells were injected into nude mice there was no formation of tumours after a minimum of 12 months (Reddel *et al*, 1988).

These cells were also maintained in continuous culture, in 75ml flasks, incubated at 37°C, 5% CO₂ in LHC-9 medium which contained penicillin (100µg.ml⁻¹) and streptomycin (50µg.ml⁻¹). When required for the assays, a confluent monolayer of BEA-2B cells was washed twice with PBS and the cells were detached using 50:50 Trypsin-EDTA:Versene solution. The cells were then washed in LHC-9 containing 10% FCS and resuspended in LHC-9 medium. This suspension of BEA-2B was used in the detachment and monoclonal antibody labelling assays and were visualised using light microscopy transmission and scanning electron microscopy.

(c) Isolation of Rat Type II Epithelial Cells

Type II alveolar epithelial cells were isolated using the method of Richards *et al* (1987), from specific pathogen-free, adult male, Wistar rats (200-250g). The rats were anaesthetised intraperitoneally with Nembutal (50mg.kg⁻¹. body wt), and the lungs perfused *in situ* with saline (0.15M, 37°C). The lungs and heart were then removed and the bronchialveolar leucocytes retrieved by repeated pulmonary lavages with 0.15M saline. Proteolytic digestion of the lungs was carried out by intratracheal instillation of 0.25% (wt./vol.) trypsin in Hanks buffered salt solution (HBSS) for 30 minutes at 37°C. The lung parenchyma was then dissected free of the trachea and bronchi and chopped into fine pieces. Digestion of the lung was stopped by the addition of 10% FCS in HBSS (Ca²⁺, Mg²⁺ free) containing Deoxyribonuclease (D'Nase, 250µg.ml⁻¹), while being shaken in a water bath at 37°C for 4 minutes. The lung debris was separated out using a 150µm filter followed by a 30µm filter. The resulting cell suspension was layered on to a Percoll discontinuous density gradient (10ml, 1.089g.ml⁻¹ Percoll: 10ml, 1.04g.ml⁻¹ Percoll: 10 ml cell suspension) and centrifuged at 250g for 20 minutes at 4°C. The cells aspirated from the 1.04/1.089 density interface, were washed with HBSS (Ca²⁺, Mg²⁺ free) containing DNase (50µg.ml⁻¹) and resuspended in MEM with the addition of gentamycin M (50µg.ml⁻¹) with 10% FCS. Viability was assessed by trypan blue exclusion and the percentage of type II cells was checked using Papanicolaou staining.

(d) Isolation of Human Type II Pneumocytes

Isolation of human type II pneumocytes was carried out using the method of Bingle *et al*, (1990). Tissue was obtained from patients whose lung or lobe had been removed for lung cancer. The tissue was taken from the

periphery of the lung to avoid major bronchi and to be as far away as possible from the tumour. Each sample of lung tissue was approximately 10g in weight.

An airway was cannulated and lung was repeatedly lavaged by instilling 50ml aliquots of 0.15M NaCl until the lung was fully inflated. The lavage process was repeated until the number of cells recovered was less than 5% of the initial cell count. The tissue was then infused with approximately 50ml of 0.5% crude trypsin (type I) in HB85 for reinflation and suspended in 0.15M NaCl at 37°C in a shaking water bath for 30 minutes. Fresh trypsin was added at regular intervals to replace that which was lost through seepage. Thereafter the tissue was chopped into 1mm pieces and suspended in HBSS (Ca^{2+} , Mg^{2+} free) containing DNase; ($250\mu\text{g}.\text{ml}^{-1}$) and FCS (10%), to prevent cell aggregation and to neutralise the enzyme. The suspension was initially filtered through sterile gauze and then through $150\mu\text{m}$ and $30\mu\text{m}$ nylon mesh. The resultant suspension was layered on to a Percoll density gradient ($1.04/1.09 \text{ gml}^{-1}$) and centrifuged at 250g for 20 minutes at 4°C. The cell layer at the 1.04ml^{-1} percoll interface was aspirated and diluted with HBSS containing DNase ($50\mu\text{g}.\text{ml}^{-1}$) and centrifuged at 250g for 20 minutes at 4°C. The resulting cell pellet was resuspended in MEM plus FCS (10%) with penicillin ($100\mu\text{g}.\text{ml}^{-1}$), streptomycin ($100 \mu\text{g}.\text{ml}^{-1}$) and gentamycin M ($50\mu\text{g}.\text{ml}^{-1}$) at a concentration of $5 \times 10^5 \text{ cells ml}^{-1}$. These cells were plated out at 2.5×10^4 in a microtitre plate and incubated at 37°C in a 5% CO_2 humidified incubator for 5-8 days with the medium changed every 24 hours. A cytospin of the cell suspension was also prepared.

Light Microscopy:- The cells were cytochemically stained for light microscopy to demonstrate alkaline phosphatase (AP) using a modified method of Miller *et al*, (1987).

Alkaline Phosphatase:- The cytopins were air dried and then stained with naphthol phosphate and fast red (10mg naphthol AS phosphate, dissolved in 40 μ l DMSO then diluted in 10ml 0.125M 2-amino-2-methyl-1-propanol buffer, pH8.9 containing 10mg fast red) for 15 minutes at room temperature. The stain was washed off with distilled water, and the cells were counterstained with 1% methylene green for 30 seconds and mounted in aqueous medium.

2.2.2. Human Bronchoalveolar Lavage Recovery and Processing

After application of local anesthetic a flexible fiberoptic bronchoscope (Olympus, Japan) was inserted and gently wedged in a subsegmental bronchus, usually in the middle lobe. Sterile saline (0.15M) warmed to 37°C, was infused through the aspiration port and collected back via the same port under gentle suction. A total of eight 30ml aliquots (240ml total) were used to lavage the site usually in the middle lobe. The bronchoalveolar lavage (BAL) was then kept on ice for the rest of the procedure. The BAL was strained through a double layer mesh of cotton gauze to trap mucous particles. The filtered BAL was then centrifuged at 250g, 4°C for 10 minutes to separate out the cells from the fluid. The resultant supernatant was recentrifuged at 1000g, 4°C for 20 minutes to remove any residual cell, after which the BAL fluid (BALF) was decanted.

2.2.3. Functional Assays

(a) Attachment Assay

A suspension of A549 epithelial cells (5×10^6 cells ml^{-1}) was incubated with ^{51}Cr (10MBq. ml^{-1}) at 37°C for 1 hour, in a shaking water bath. The cell suspension was then washed twice with serum free MEM to remove any free ^{51}Cr which had not been taken up by the cells and resuspended in MEM, with or without an antioxidant/antiprotease present, to give a final concentration of 1×10^6 cells. ml^{-1} . These suspensions were then subjected to an insult after which triplicate aliquots of treated ^{51}Cr -labelled cell suspensions (5×10^4 cells in $200\mu\text{l}$), together with appropriate controls were added to 96 well microtitre plates and incubated at 37°C in 5% CO_2 . At varying time points the supernatants were aspirated and the wells washed twice with PBS ($200\mu\text{l}$). Triton-X-100 (0.1%, $200\mu\text{l}$) was then added to lyse the cells that had attached, and the counts present in the Triton-X-100 were measured in a gamma well counter (LKB, Wallac, Finland). The total cell associated counts were measured in triplicate at each point.

$$\% \text{ attachment} = \frac{\text{c.p.m. on attached cells} - \text{background}}{\text{c.p.m. on all cell} - \text{background}} \times 100$$

(b) ^{51}Cr Release

In order to determine the level of spontaneous ^{51}Cr release from A549 cells with time in the attachment assay, ^{51}Cr epithelial cells were plated out in MEM as in the attachment assay. At set time points the supernatant was collected and each well was then washed twice with PBS ($200\mu\text{l}$). These washings were combined with the initial supernatant and centrifuged at 400g for 10 minutes. The supernatants from these washes were decanted and the cell pellet of unattached cells was lysed with Triton-X-100 ($200\mu\text{l}$). Cells attached to the plate were lysed with Triton-

X-100 (200 μ l) and were then combined with the lysate of the unattached cells. The counts in the cell lysates were used as a measure of the ^{51}Cr label remaining on the cells. The counts in the cell free supernatant combined with washings were used as a measure of the ^{51}Cr label released from the cells over the course of the experiment.

(c) Detachment Assay

Cell Lines

Confluent monolayers of A549 or BEA-2B epithelial cells were trypsinised off the 75ml flasks as previously described. The cells were resuspended in MEM containing 10% FCS for A549 and LHC-9 for BEA-2B and labelled with 0.37MBq.ml ^{51}Cr in PBS to give a final concentration of 5×10^5 cells ml.⁻¹. The labelled cells were plated out in a 96 well microtitre plate (5×10^4 cells. well⁻¹), and allowed to adhere overnight in an incubator at 37°C, in a humidified atmosphere with 5% CO₂ added.

Primary Type II Cell

After isolation the rat type II cells (RTTC) were plated out at 5×10^5 cell well⁻¹ in a 96 well tissue culture plate and incubated at 37°C, with 5% CO₂. After 3 days the RTTC cells were washed twice and the medium replaced with MEM and 10% FCS containing Chromium (0.37 MBq.ml⁻¹ ^{51}Cr .) in 200 μ l. The plate was then incubated overnight and used in the detachment assay.

After plating the cells out overnight in medium, the supernatants were removed and the wells washed twice with PBS (200 μ l). This removed any excess free ^{51}Cr which had not been taken up by the cells overnight. Serum free medium (200 μ l) containing the intervention was added to the required number of wells. To one set of triplicate wells 0.1% Triton-X-

100 (200 μ l) was added to produce 100% lysis as a positive control, measured as total cell associated cpm. The plates were incubated for up to 4 hours at 37°C with 5% CO₂ and then centrifuged at 100g for 5 minutes. Fifty μ l of the cell free upper half of the supernatant was removed and counted to give a measure of cell lysis (X) when calculated as below. The remainder of the supernatant containing detached cells was aspirated and combined with two washes of PBS (200 μ l) from each well (y); this when calculated as below gave a measure of cell detachment (Donaldson *et al*, 1988). So the equations for calculating the final results are:-

$$\text{Lysis} = 4 \times X \text{ c.p.m.}$$

$$\text{Detachment} = y - (3 \times X) \text{ c.p.m.}$$

(d) Uptake of [³H] Thymidine

A549 cells were trypsinized off subconfluent flasks and resuspended in complete medium. These cells were incubated overnight at 5 x 10⁴ cells.well⁻¹ in a microtitre plate at 37°C, with 5% CO₂, to allow adherence. The wells were washed twice with PBS (200 μ l.well⁻¹) to remove any non-adherent cells. Medium containing the intervention or the appropriate controls were added in triplicate wells along with [³H] thymidine (0.01MBq. 20 μ l.well⁻¹). The cell nuclei were harvested 16 hours later with a miniwash 2000 cell harvester (Dynatech, Billingshurst, U.K.) onto glass fibre paper. The uptake of [³H] thymidine was measured in a PW 4700 liquid scintillation counter (Phillips, Eindhoven, Netherlands).

(e) Spreading Assay

This assay is an adaptation of the method of Donaldson *et al*, (1984) with

epithelial cells substituted for macrophages. Confluent monolayers of A549 epithelial cells were trypsinised off the 75ml flasks as previously described. The cells were resuspended at 5×10^6 cells ml^{-1} in MEM containing 10% FCS. This suspension was pipetted out into 8 Culture Chamber slides ($400\mu\text{l.well}^{-1}$, (GIBCO) and the cells allowed to adhere for 24 hours at 37°C in 5% CO_2 incubator.

The MEM with 10% FCS was removed and the slides washed twice with PBS ($400\mu\text{l}$) to remove any non-adherent cells. Serum less medium ($400\mu\text{l}$) containing the intervention or appropriate controls (10%) were added in duplicate to each chamber slide. The slides were then incubated at 37°C in 5% CO_2 . At varying time points (0.5 - 4 hours) the medium was removed, the slides washed twice in PBS to remove the non-adherent cells and the adherent cells were fixed in alcohol (90%).

The cells were then stained with Diff-Quik using a standard procedure. Measurement of the cells maximum diameter was carried out using a Tektronix 4050 series computer linked to a digitising tablet (GIS, Blairgowrie, UK) for a total of 400 cells for each condition and each time point, taken from three experiments. The maximum diameter was taken as the distance between the two furthest edges of the cytoplasm.

2.2.4. Cigarette Smoke Exposure

For the attachment assay aliquots, of ^{51}Cr -labelled cells in 2ml of MEM were exposed to smoke while being agitated in a tonometer (Brown *et al*, 1991) consisting of a siliconised glass (a) flask in a water bath attached to a four channel smoking machine (b) (figure 2.1). This technique allows a thin layer of cells to be exposed to cigarette smoke, obtained from a medium tar cigarette (G4), produced by a smoking machine. This system was set up with a 37ml per puff volume which is the equivalent

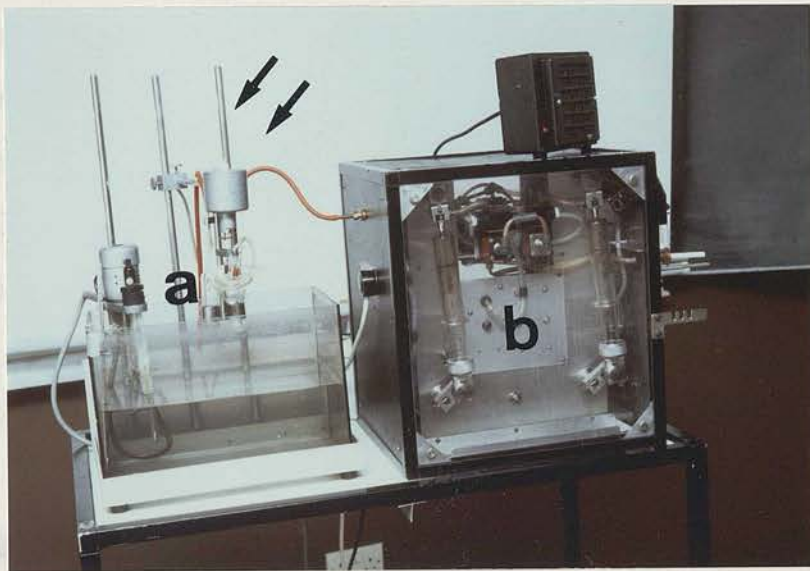


Figure 2.1. Cigarette smoke exposure system. This consists of a siliconised glass flask (a) in a tonometer system (arrow) attached to a four channel smoking machine (b). This comprises four glass syringes fitted with 2-way valves driven by a motor.

(DMSC) of a known volume (10ml) was then used to remove the particles under a gentle vacuum. This extract was then stored at -70°C until required, and was stable for up to four weeks. The extract was diluted to 0.1, 0.5, 1, 2 and 3% of the original concentration with MEM for the detachment and proliferation assays.

(b) Aqueous Extraction

Aqueous cigarette smoke condensates (CSC) were produced by two methods: (1) in the method of Holden *et al.*, (1989) whole or vapour phase cigarette smoke from three medium tar cigarettes was bubbled through 3ml of HBSS. The system to facilitate this was a three-way stopcock with a cigarette inserted in a holder in one arm, a 10ml syringe

volume per puff of the average cigarette smoker. Vapour phase cigarette smoke was produced by drawing whole smoke through a Cambridge filter to remove particulate material (Guerin, 1980). Control cells were sham exposed in the tonometer, in room air, for the same length of time. The cell samples were washed twice immediately after smoke exposure, in MEM at 200g for 15 minutes to remove any soluble condensate that may have formed with the components of the cigarette smoke dissolving in the medium. The cells were resuspended at $2.5 \times 10^5 \text{ ml}^{-1}$ in MEM with 10% FCS. These cells were used in the attachment assay.

2.2.5. Cigarette Smoke Condensate

Two different types of cigarette smoke condensate were produced.

(a) Organic Extraction

Standard medium tar cigarettes were smoked through a Cambridge filter to deposit 200mg of tobacco particles onto the filter. Dimethylsulfoxide (DMSO) of a known volume (10ml) was then used to remove the particles under a gentle vacuum. This extract was then stored at -70°C until required, and was stable for up to four weeks. The extract was diluted to 0.1, 0.5, 1, 2 and 3% of the original concentration with MEM for the detachment and proliferation assays.

(b) Aqueous Extraction

Aqueous cigarette smoke condensates (CSC) were produced by two methods: (1) in the method of Holden *et al*, (1989) whole or vapour phase cigarette smoke from three medium tar cigarettes was bubbled through 3ml of HBSS. The system to facilitate this was a three-way stopcock with a cigarette inserted in a holder in one arm, a 10ml syringe

in another arm and in the third arm, a 5ml glass pipette. By switching the three-way stopcock, cigarette smoke could be drawn into the syringe and then bubbled through the HBSS (s); (2) an adaptation of this method was also used where whole or vapour phase cigarette smoke from 3 cigarettes was passed over 3ml of HBSS agitated in the tonometer system (t).

The condensate was made up fresh on the morning of each experiment and this solution was then diluted to give concentration range of 0.1, 1, 2, 5, 10 and 20% of the original extract.

The condensates were used in the functional assays of detachment, proliferation and spreading and the assay to quantify F-actin.

2.2.6. ECM Coating of Tissue Culture Plates

Type I and IV collagen, both from human placenta were prepared by dissolving 10mg of collagen in 0.1N acetic acid (10ml.) and mixed thoroughly. Both fibronectin (Human placenta) and BSA were dissolved in PBS, again to give stock solutions of 1mg.ml^{-1} . Laminin again from human placenta was dissolved in Tris NaCl buffer to give a stock concentration of 1mg.ml^{-1} .

These stock solutions were then diluted with sterile H_2O_2 to give final concentrations of 1mg.ml^{-1} and sterilised by filtering through a $0.1\mu\text{m}$ filter. These were then plated out, in $100\mu\text{l}$ aliquots for each well in a 96-well plate and incubated at 37°C to dryness for 2-3 days. The plates were then washed once with sterile water and stored at 4°C until required.

2.2.7. F-Actin Content

The F-actin content of the epithelial cell was examined for both quantity and distribution.

(a) Quantification

FITC-phalloidin, a fluorescent derivative of the phallotoxin from *Amanita phalloides* which binds with high affinity to F-actin, was used to assess cellular F-actin by flow cytometry.

The method was adapted from Howard & Meyer (1984). The epithelial cells were trypsinized off the culture flasks as previously described, washed (200g, 10 min.) and resuspended in MEM + 10% FCS. The cell suspension was added to teflon wells for 1 hour. Thereafter the cells were washed twice with MEM and resuspended to give a final concentration of 1×10^6 cells ml^{-1} . A549 epithelial cells were exposed to treatment, after which the cells were washed, fixed, permeabilized and stained in a single step. This was achieved by adding 0.9ml of 3.7% phosphate-buffered formalin, containing 1.65×10^{-7} M FITC-phalloidin, and lysophosphatidyl choline at $100\mu\text{g}.\text{ml}^{-1}$ to 0.1 ml aliquots of the treated cells and incubating at 37°C for 15 minutes. The cells were then spun down at 250g for 10 minutes and resuspended in fresh phosphate-buffered formalin.

EPICS Analysis of FITC-phalloidin Stained Cells:- The fixed cells were stored at 4°C in the dark until required. The analysis of a minimum of 5000 cells was carried out using an EPICS Profile II (Coulter Electronics, Luton, UK). The cells were excited with an argon laser at 488nm and emission was read at 522nm. The data of forward and side light scatter signals were stored with histograms of fluorescence and log fluorescence. The analysis was gated on non-aggregated cells only.

(b) Visualisation

Rhodamine-Phalloidin Staining:- The acetone-fixed epithelial cells

were washed twice with PBS, containing 0.2% BSA fraction V and 0.1% azide and then stained with the rhodamine-conjugated phalloidin in PBS which specifically binds to F-actin, at a $0.15\mu\text{mol}$ concentration for 20 minutes. The slides were washed twice with the PBS and then mounted with a 1:1 solution of PBS. The edges of the coverslips were sealed with glyceel. The specimens were viewed on a photomicroscope (Leitz, Germany).

2.2.8. Monoclonal Antibody Labelling

Epithelial cells were labelled with mouse and rat monoclonal antibodies to the specific cell integrins, CDw49b, CDw49d, CDw49e, CDw49f, CD29, α_v , $\alpha_v\beta_3$ and the irrelevant mAb 52 μ using a standard indirect immunofluorescence protocol as described later.

Protocol:- Untreated/treated cells (2×10^5) were added to the prerequisite number of wells in a strip of flexi-wells and centrifuged at 250g for 1 minute. The supernatant was discarded and the cells resuspended. To these cells was added 50 μl of each antibody at the relevant dilutions and for each condition a control of 50 μl of wash buffer (PBS + 0.2% bovine serum albumin + 0.1% azide) only was added to a well of the treated cells. The cells were left on ice for 30 minutes incubation, after which time 120 μl of wash buffer was added to each well. The plates were spun at 250g for 1 minute, the supernatant discarded and the cells resuspended. The second antibody, in all cases except CDw49f, Fab'FITC labelled anti-mouse (1/25 dilution) and for CDw49f, Fab'FITC labelled anti-rat (1/25 dilution) was then added to the cells (50 μl aliquot). These were again incubated for 30 minutes and then 120 μl of wash buffer added. The plates were spun at 250g for 1 minute, the supernatant

discarded and the wash repeated. The cells were resuspended in 150 μ l of wash-buffer and combined with 150 μ l of fixative (37% formaldehyde 5ml + 85ml of PBS). The mean and log fluorescence are then read on the EPIC PROFILE II flow cytometer as before.

2.3. STATISTICAL ANALYSIS

The data was analysed using the SPSS/PC+ computer package. Either Oneway analysis of variance or ANOVA was performed to compare multiple treatments and to determine possible differences between means.

EFFECTS OF CIGARETTE SMOKE AND ITS CONDENSATES
ON EPITHELIAL CELL FUNCTION IN VITRO

CHAPTER 3-9

RESULTS

In this Chapter the effects of cigarette smoke and its condensates and the oxidant hydrogen peroxide on the functional aspects of epithelial cells are studied. The methods used for the measurement of $[^{14}C]$ and $[^3H]$ uptake were described in Chapter 2. The C3A/21 epithelial cell line was also used and the primary culture and type II cells were used in certain experiments to validate the use of the A149 epithelial cells.

It was initially necessary to determine the levels of cigarette smoke to which epithelial cells should be exposed to mimic *in vivo* exposure.

3.1 ASSESSMENT OF CIGARETTE SMOKE EXPOSURE

It is possible to assess the number of cigarettes smoked by simply measuring the Carboxyhaemoglobin (COHb) levels in human blood samples from smokers. It was this measurement that was used to estimate the degree of cigarette smoke exposure of cells in the gas phase of cigarette smoke in the incubator system. The pH of the medium alone was tested after cigarette exposure, since a change in the pH would have a direct effect on the cells when exposed to the cigarette smoke. In addition, the degree of condensation of the cigarette smoke was estimated by measuring the amount of water in the medium after exposure.

CHAPTER 3

EFFECTS OF CIGARETTE SMOKE AND ITS CONDENSATES ON EPITHELIAL CELL FUNCTION IN VITRO

In this Chapter the effects of cigarette smoke, its condensates and the oxidant hydrogen peroxide, on the functional assays of epithelial attachment, detachment and [^3H] uptake were examined. The cell line used for the majority of the experiments was the A549 epithelial cell line. The BEA-2B epithelial cell line was also used and the primary culture rat type II cells were used in certain experiments to attempt to validate the use of the A549 epithelial cells.

It was initially necessary to determine the levels of cigarette smoke to which epithelial cells should be exposed to mimic in vivo exposure.

3.1. ASSESSMENT OF CIGARETTE SMOKE EXPOSURE

It is possible to assess the number of cigarettes smoked by simply measuring the Carboxyhaemoglobin (CoHb) levels in venous blood samples from smokers. It was this measurement that was used to estimate the degree of cigarette smoke exposure of cells to the gas phase of cigarette smoke in the tonometer system. The pH of the medium alone was tested after cigarette exposure, since a change in the pH could have a detrimental effect on the cells when used in the attachment assay. In addition, the cigarette smoke produced by the exposure system underwent a crude assessment for the production of oxygen radicals.

3.1.1. CoHb Levels During In Vitro Cigarette Smoke Exposure

The degree of cigarette smoke exposure that the cells were to receive was estimated by measuring the CoHb levels reached when aliquots of whole blood (2ml) were exposed to varying amounts of cigarette smoke, in the same fashion as cells in the tonometer system (Figure 2.1).

Results:- Table 3.1 gives the percentage of CoHb in aliquots of blood exposed to 1,2,4 or 6 puffs of whole and vapour phase smoke. The CoHb levels achieved for whole and vapour phase smoke are approximately equal on a puff to puff basis, with 4 puffs of cigarette smoke giving a CoHb level observed in a venous blood sample of a very heavy cigarette smoker (Prignot, 1987). This was the factor which determined the use of 4 puffs as the standard exposure for epithelial cells in further experiments.

3.1.2. Effect of Cigarette Smoke Exposure on pH

Cigarette smoke has the potential to alter the pH of the medium in which the cells are exposed, which in turn may effect the following attachment assays. The medium MEM (2ml) was exposed to 4 puffs of either whole or vapour phase cigarette smoke using the system described in Section 2.2.4, and the pH measured using a Gallenkamp pH stick (Loughborough, UK).

Results:- Compared with the control solution of MEM where the pH was 7.66 there was little change when MEM was exposed to whole cigarette smoke, pH 7.45, or to vapour cigarette smoke, pH 7.56. This would have no effect on the cells in the attachment assay.

3.1.3 Oxygen Radical Production in Cigarette Smoke

As has previously been discussed, Fryer and colleagues (1983) have measured the oxidative potential of cigarette smoke using a whole spin

| Concentration (%) | HBSS | pH Whole | Vapour |
|-------------------|-------------|-------------|-------------|
| 0.1 | 7.48 (0.13) | 7.47 (0.20) | 7.59 (0.08) |
| 1 | 7.50 (0.11) | 7.56 (0.15) | 7.56 (0.13) |
| 2 | 7.54 (0.11) | 7.53 (0.77) | 7.55 (0.10) |
| 5 | 7.49 (0.14) | 7.54 (0.11) | 7.49 (0.07) |
| 10 | 7.50 (0.16) | 7.51 (0.13) | 7.50 (0.13) |
| 20 | 7.51 (0.15) | 7.49 (0.13) | 7.53 (0.12) |

Table 3.4. The effect of increasing concentrations of HBSS, whole CSC or vapour CSC on the pH of culture medium, MEM. The data represents the mean (SEM) of three separate experiments in each case. There were no significant changes in the pH of the medium.

solution of ferricytochrome C and was determined spectrophotometrically (Pye Unicam SP8-400 Spectrophotometer, Cambridge, UK) over a range of wavelengths (580 - 520nm) measuring the peak at 550nm. The amount of reduced ferricytochrome C was calculated based on an extinction coefficient of 21,000dm²mol⁻¹ for ferricytochrome C. The amount attributable to O₂ production was calculated as follows:

$$O_2 = \frac{X - X_{\text{min}}}{\epsilon \cdot l} \cdot \frac{1}{2}$$

Results:- These results are summarised in Table 3.2 for whole smoke exposure. There was little or no reduction of ferricytochrome C with a

3.1.3. Oxygen Radical Production by Cigarette Smoke

As has previously been discussed Pryor and colleagues (1983) have measured the oxidative potential of cigarette smoke using electron spin resonance. We used a less sensitive system to attempt to measure the production of oxygen radicals, in particular $O_2^{\cdot -}$ and H_2O_2 , in fluids exposed to smoke within the exposure system.

(a) Superoxide Radical Production by Cigarette Smoke

The standard method for measuring $O_2^{\cdot -}$ production from cells is by the SOD-inhibitable reduction of ferricytochrome C (Babior *et al*, 1973) which is the method used below.

Protocol:- Inclusion of the enzyme SOD eliminated all ferricytochrome C reduction by agents other than $O_2^{\cdot -}$. This assay was used by exposing a solution of ferricytochrome C ($80\mu M$, 2ml) with (x_{SOD}) or without (x) SOD ($75,000$ unit ml^{-1} ,) to 1, 2 or 4 puffs of whole or vapour phase cigarette smoke in the tonometer. The difference in absorbance of the smoke-exposed ferricytochrome C solutions was compared to a control solution of ferricytochrome C and was determined spectrophotometrically (Pye Unicam SP8-400 Spectrophotometer, Cambridge, UK) over a range of wavelengths (580 - 520nm) measuring the peak at 550nm. The amount of reduced ferricytochrome C was calculated based on an extinction co-efficient of $21.0nM^{-1}cm^{-1}$ for ferricytochrome C. The amount attributable to $O_2^{\cdot -}$ production was calculated as follows:

$$X - X_{SOD} = O_2^{\cdot -}$$

Results:- These results are summarised in Table 3.2 for whole smoke exposure. There was little or no reduction of ferricytochrome C with a



| Exposure (puffs) | Reduced ferricytochrome C (nmoles) | | | |
|------------------|------------------------------------|---------|--------------------|---------|
| | x | | x-x _{SOD} | |
| sham | 0.62 | (0.88) | 0.53 | (0.74) |
| 1 | 33.08 | (17.15) | 22.02 | (21.95) |
| 2 | 30.21 | (4.36) | 2.54 | (2.37) |
| 4 | 50.64 | (6.00) | 1.52 | (2.15) |

| Exposure (puffs) | Reduced ferricytochrome C (nmoles) | | | |
|------------------|------------------------------------|--------|--------------------|--------|
| | x | | x-x _{SOD} | |
| sham | 0.85 | (1.06) | 0.32 | (0.64) |
| 1 | 1.61 | (0.39) | 0.38 | (0.27) |
| 2 | 1.60 | (0.74) | 0.56 | (0.74) |
| 4 | 2.17 | (0.64) | 0 | (0) |

Table 3.2. The effect of increasing exposure of ferricytochrome C (with/without SOD) to a) whole and b) vapour phase cigarette smoke, measured in nmoles. The data represents a mean (SEM) of triplicate samples of separate experiments.

solution which had been sham exposed in air (0.62 ± 0.88 nmoles). On exposure to whole cigarette smoke the values for reduced ferricytochrome C ranged from 14.09 - 59.02 nmoles, with an average of 33.08 nmoles for 1 puff, the average was 30.21 nmoles for 2 puffs with a range of 25.89 - 47.22 nmoles and an average of 50.64 nmoles for 4 puffs with a range of 45.31 - 59.02. The addition of SOD to the ferricytochrome C solution prior to whole cigarette smoke exposure decreased the levels of reduced ferricytochrome C due to $O_2^{\cdot-}$ production to between 0.56 - 3.05 nmoles (Table 3.2). Vapour phase cigarette smoke exposure produced minimal increases in reduced ferricytochrome C at all levels of exposure (1.60 - 2.17 nmoles) and this was almost completely abolished by the addition of SOD (0 - 0.56 nmoles). Thus both whole and vapour cigarette smoke in the exposure system generated reactive radicals which can produce reduced ferricytochrome C, the majority of this was not the result of $O_2^{\cdot-}$ production.

(b) Hydrogen Peroxide Production by Cigarette Smoke

The production of H_2O_2 during *in vitro* smoke exposure was estimated using the method of Pick & Keisari (1980).

Protocol:- The "phenol red" reaction mixture containing potassium buffer with Type II, salt-free horse radish peroxidase (5mg.ml^{-1}), phenol red (0.028M) and sodium chloride (140nM) was exposed to whole or vapour phase cigarette smoke as described in Section 2.2.4. A standard curve of dilutions of a reference solution of H_2O_2 ($1\text{-}50\mu\text{M}$) was also prepared at the same time. After cigarette smoke exposure the colour reaction was completed with the addition of 1N sodium hydroxide (NaOH). Absorbance of the treated phenol red solutions were then read at 610 nm on the same spectrophotometer used for the $O_2^{\cdot-}$ assay and compared with

the absorbance curve derived from the standard solutions of H_2O_2 .

Results:- There was H_2O_2 production from both whole and vapour phase cigarette smoke using the exposure system. The amount generated from whole cigarette smoke was $1\text{-}5\mu\text{M}$ and $0.5\text{ - }2.8\mu\text{M}$ from vapour phase cigarette smoke (Table 3.3). These values are at the lower end of the standard curve (range $1\text{ - }50\mu\text{M}$) and thus the accuracy may be questionable.

The exposure system produces relevant physiological levels of cigarette smoke containing reactive radicals. Moreover, cigarette smoke exposure does not alter the pH of the test medium.

3.2. A549 EPITHELIAL CELL ATTACHMENT

Only the A549 epithelial cells were used in the following attachment assay.

3.2.1. Time Course of Attachment of A549 Epithelial Cells Following Exposure to Cigarette Smoke

The time course of attachment of A549 epithelial cells to plastic after exposure to either whole or vapour phase cigarette smoke was examined over 24 hours.

Protocol:- Suspensions of A549 epithelial cells ($1 \times 10^6\text{ml}^{-1}$, 2ml aliquots) were radiolabelled with ^{51}Cr as described in Section 2.2.3a., and then exposed to 4 puffs of either whole or vapour cigarette smoke as described in Section 2.2.4. These ^{51}Cr labelled cells were then used in the attachment assay (Section 2.2.3a.) and incubated at 37°C , 5% CO_2 for up to 24 hours. At varying time points triplicate aliquots of cells of each

| Exposure (puffs) | Whole | | Vapour | |
|------------------|------------------------------------|--------|--------|--------|
| | H ₂ O ₂ (μM) | | | |
| sham | 0 | (0) | 0 | (0) |
| 1 | 1.02 | (0.7) | 2.70 | (1.58) |
| 2 | 2.25 | (2.02) | 0.55 | (0.71) |
| 4 | 4.84 | (2.62) | 1.26 | (1.21) |

Table 3.3. The effect of increasing exposure of "phenol red" solution to either whole or vapour phase cigarette smoke, measured in uM. The data represents a mean (SEM) of triplicate samples of four separate experiments.

3.3.2. Dose Effect of Cigarette Smoke Exposure on A549 Epithelial Cell Attachment

The previous experiment showed that a fixed dose of cigarette smoke reduced A549 epithelial cell attachment. The following experiment

treatment were harvested and the cell-associated c.p.m. measured as an indication of cell attachment. Epithelial cell viability remained >95% immediately after smoke exposure, as assessed by trypan blue exclusion.

Results:- A549 epithelial cells demonstrated a decreased ability to attach to plastic over a period of 24 hours, after exposure to 4 puffs of either whole or vapour phase cigarette smoke (Figure 3.1). This effect was significant after 45 minutes, and was similar ($p > 0.05$) following exposure to both whole and vapour phase cigarette smoke, when compared with sham exposed cells (Figure 3.1.). At 4 hours $23.5 \pm 4.9\%$ of the unexposed cells had attached, while only $4.3 \pm 1.8\%$ of the whole and $4.1 \pm 1.4\%$ of the vapour phase cigarette smoke exposed cells had attached. Cell attachment of control, sham exposed and cigarette smoke exposed cells continued to increase up to 20 hours and this value then leveled out at 24 hours. Sham exposure produced by agitating cells in the tonometer in air did not affect the ability of cells to attach when compared with untreated cells.

As there seemed to be no differences in effect of either whole or vapour phase cigarette smoke on cell attachment, further experiments were carried out using vapour phase cigarette smoke, as this was much cleaner to use and is representative of the deep lung exposure. The experiments were also limited to a 4 hour incubation, as this was considered long enough to demonstrate any difference.

3.2.2. Dose Effect of Cigarette Smoke Exposure on A549 Epithelial Cell Attachment

The previous experiment showed that a fixed dose of cigarette smoke reduced A549 epithelial cell attachment. The following experiment

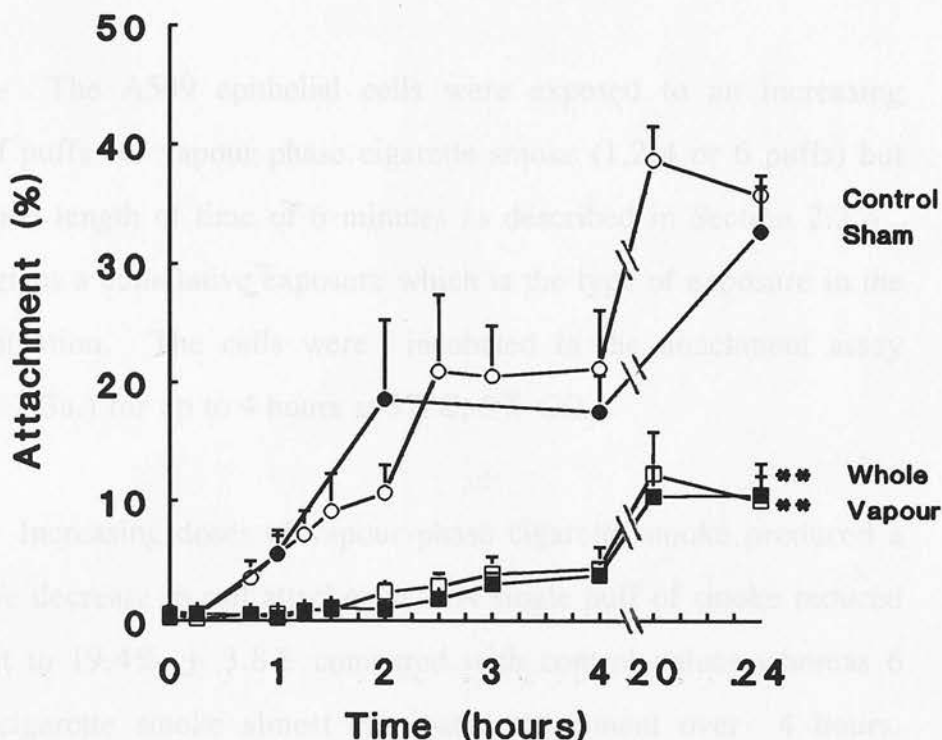


Figure 3.1. The effect of either 4 puffs of whole (■) or vapour (□) phase cigarette smoke exposure on the attachment of A549 epithelial cells to culture plastic compared with control (○) or sham exposed (●) A549 epithelial cells. The data is the mean of triplicate wells in three separate experiments and the bars indicate 1 SEM. Smoke exposed cells showed significantly reduced attachment from 45 minutes. ** $p < 0.01$ when compared with control or sham exposed cells.

examined the effect of increasing doses of cigarette smoke on A549 epithelial cell attachment.

Protocol:- The A549 epithelial cells were exposed to an increasing number of puffs of vapour phase cigarette smoke (1,2,4 or 6 puffs) but for the same length of time of 6 minutes as described in Section 2.2.4., this then gives a cumulative exposure which is the type of exposure in the in vivo situation. The cells were incubated in the attachment assay (Section 2.2.3a.) for up to 4 hours at 37°C, 5% CO₂.

Results:- Increasing doses of vapour phase cigarette smoke produced a progressive decrease in cell attachment. A single puff of smoke reduced attachment to 19.4% \pm 3.8% compared with control values whereas 6 puffs of cigarette smoke almost eliminated attachment over 4 hours. The difference in attachment following smoke exposure became significant ($p > 0.01$) after 2 hours following 4 and 6 puffs of smoke (Figure 3.2).

3.2.3. ⁵¹Cr Release from A549 Epithelial Cell Exposed to Cigarette Smoke

Changes in cell attachment described above, in response to cigarette smoke, could be a result of the release of radiolabel from attached cells, thus falsely underestimating attachment. To answer this question the levels of cell-associated and free ⁵¹Cr label were measured as described in Section 2.2.3b. in cultured cells that had been exposed to cigarette smoke.

Protocol:- Two sets of experiments were carried out. The first using a

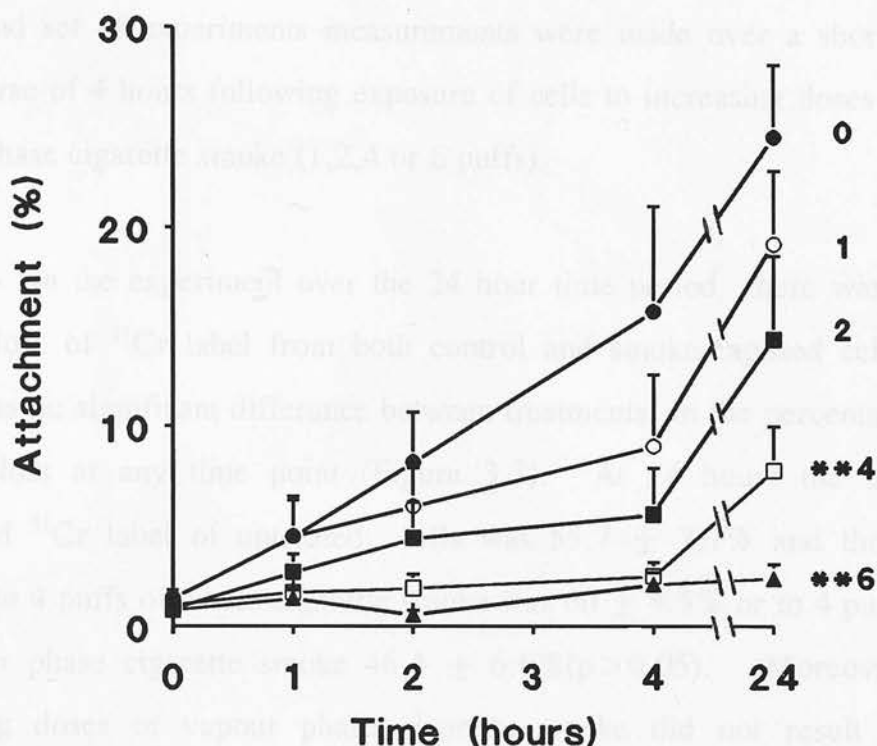


Figure 3.2. The effect of increasing vapour phase cigarette smoke exposure, sham (●), 1 puff (-○-), 2 puffs (-■-), 4 puffs (-□-), and 6 puffs (-▲-) on attachment of A549 epithelial cells to culture plates over 24 hours, measured as a percentage of the total cells plated out. The data is the mean of triplicate wells in three separate experiments and the bars indicate 1 SEM. ** $p < 0.01$ when compared with sham exposure.

fixed exposure of 4 puffs of whole and vapour phase cigarette smoke with measurements of free and cell associated ^{51}Cr measured over 24 hours; in the second set of experiments measurements were made over a shorter time course of 4 hours following exposure of cells to increasing doses of vapour phase cigarette smoke (1,2,4 or 6 puffs).

Results:- In the experiment over the 24 hour time period there was a gradual loss of ^{51}Cr label from both control and smoke-exposed cells. There was no significant difference between treatments in the percentage of ^{51}Cr loss at any time point (Figure 3.3). At 24 hours the cell associated ^{51}Cr label of untreated cells was $55.7 \pm 7.7\%$ and those exposed to 4 puffs of whole cigarette smoke was $60 \pm 4.5\%$ or to 4 puffs of vapour phase cigarette smoke $46.4 \pm 6.6\%$ ($p > 0.05$). Moreover, increasing doses of vapour phase cigarette smoke did not result in significant loss of ^{51}Cr at 24 hours, when compared with control cells (Figure 3.4). All subsequent attachment assays were incubated for a maximum of 4 hours at which time there was minimal loss of ^{51}Cr label from the cells.

3.2.4. A549 Epithelial Cell Attachment After Exposure to Hydrogen Peroxide

To examine the contention that the effect of cigarette smoke on epithelial attachment was oxidant-mediated, the effects of the oxidant H_2O_2 on cell attachment were studied. Duane and co-workers (1991) used 0.1 to 10mM H_2O_2 as an oxidant stress in bovine pulmonary endothelial cells and it was this range of concentrations that were used in the following assays.

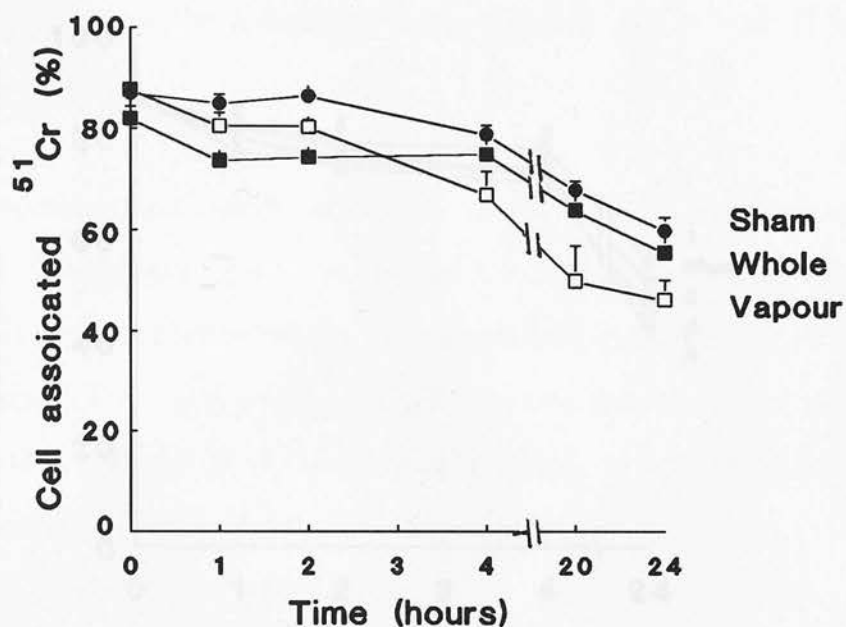


Figure 3.3. The effect of either 4 puffs of whole (■) or vapour phase (□) cigarette smoke exposure and sham exposure (●) on the release of ^{51}Cr label from A549 epithelial cells over 24 hours, expressed as percentage of ^{51}Cr that is cell associated. The data is the mean of triplicate wells in three separate experiments and the bars indicate 1 SEM in each case. There were no significant differences between types of exposure.

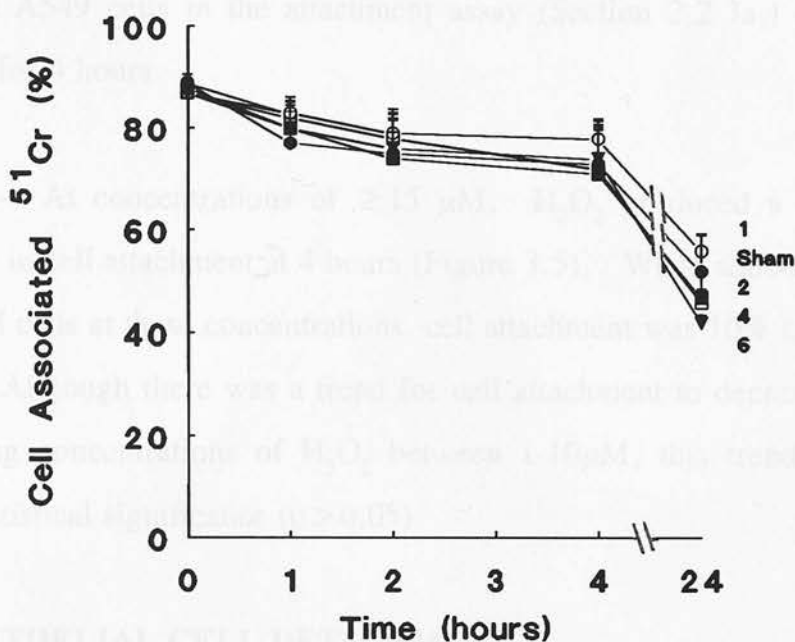


Figure 3.4. The effect of increasing vapour phase cigarette smoke exposure, sham (●), 1 puff (○), 2 puffs (■), 4 puffs (□), and 6 puffs (▲) on the release of ^{51}Cr label from A549 epithelial cells over 24 hours, expressed as percentage of ^{51}Cr that is cell associated. The data is the mean of triplicate wells in three separate experiments and the bars indicate 1 SEM. There were no significant differences between levels of exposure.

Protocol:- The H_2O_2 was diluted in MEM on the morning of each experiment to produce 5,10,15, 20 and $50\mu\text{M}$ H_2O_2 . This was incubated with the A549 cells in the attachment assay (Section 2.2.3a.) at 37°C , $5\%\text{CO}_2$ for 4 hours.

Results:- At concentrations of $\geq 15\mu\text{M}$, H_2O_2 produced a dramatic decrease in cell attachment at 4 hours (Figure 3.5). When added to A549 epithelial cells at these concentrations, cell attachment was 10% of control values. Although there was a trend for cell attachment to decrease with increasing concentrations of H_2O_2 between $1\text{-}10\mu\text{M}$, this trend did not reach statistical significance ($p > 0.05$).

3.3. EPITHELIAL CELL DETACHMENT

The effects of the three types of cigarette smoke condensate and hydrogen peroxide on epithelial detachment were studied. Although the epithelial cells *in vivo* are acutely subjected to cigarette smoke, which contains reactive radicals with a very short half life, cigarette smoke forms condensate in the ELF of the lungs which is thought to redox for prolonged periods of time (Pryor, 1986), prolonging potential for injury to epithelial cells. For this reason cigarette smoke condensate was used in the epithelial detachment assay. The organic condensate was made from the non-gaseous particles generated from whole smoke, which were trapped on a Cambridge filter and required dissolution with an organic solvent (Section 2.2.5a.). Whole smoke condensate was prepared from unfiltered cigarette smoke and diluted in MEM; vapour smoke condensate consisted of the filtered gaseous phase of whole smoke (Section 2.2.5b.) and this was also diluted in MEM. These latter two condensates were produced in an aqueous solution as certain organic solvents can act as

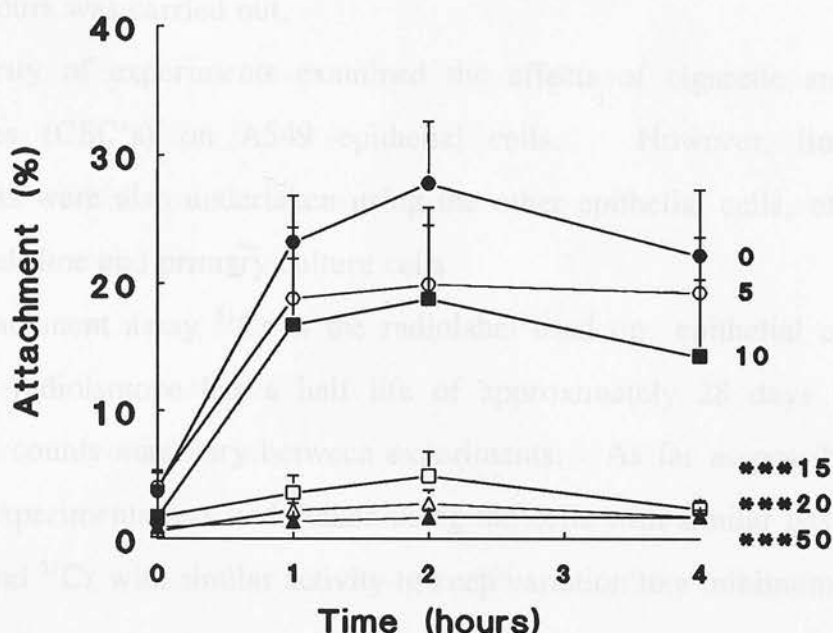


Figure 3.5. The effect of increasing concentrations of H_2O_2 , none (●), 5uM (○), 10uM (■), 15uM (□), 20uM (▲), and 50uM (△) on the attachment of A549 epithelial cells to culture plates over 4 hours, measured as a percentage of the total cells plated out. The data is the mean of triplicate wells in four separate experiments and the bars indicate 1 SEM. ** $p < 0.01$ compared with medium alone (0).

The organic solvent used for extraction was DMSO. When tested for cytotoxicity DMSO was found to cause lysis of A549 epithelial cells at concentrations of 2% and greater. This limited the concentration of organic solvents that could be added.

Procedures: Organic CSC was dissolved in MEM to give final concentrations of 0.1, 0.5, 1, 2 and 3%. These solutions were incubated at 37°C, 5% CO_2 for 4 hours in the presence of cells (Section 3.2.3c). Cells were then

oxygen radical scavengers (McDevitt, 1989). The standard end point at which detachment was assayed was at 4 hours, although a time course of up to 24 hours was carried out.

The majority of experiments examined the effects of cigarette smoke condensates (CSC's) on A549 epithelial cells. However, limited experiments were also undertaken using the other epithelial cells, of the BEA-2B cell line and primary culture cells.

In the detachment assay ^{51}Cr is the radiolabel used on epithelial cells. Since this radioisotope has a half life of approximately 28 days total number of counts may vary between experiments. As far as possible a series of experiments was undertaken using the cells with similar passage numbers and ^{51}Cr with similar activity to keep variation to a minimum.

There was the possibility that the CSC could change the pH of the assay medium (MEM), and so affect the functional properties of the epithelial cells. However, MEM contained the pH indicator phenol red which to the naked eye did not change to an acidic or alkalitic colour with the addition of any of the condensates.

3.3.1. Effect of Organic C.S.C. on A549 Epithelial Cell Detachment

The organic solvent used for extraction was DMSO. When tested for cytotoxicity DMSO was found to cause lysis of A549 epithelial cell monolayer at concentrations of 5% and greater. This limited the concentration of organic condensate that could be studied.

Protocol:- Organic CSC was diluted in MEM to give final concentrations of 0.1, 0.5, 1, 2 and 3%. These solutions were incubated at 37°C, 5% CO_2 for 4 hours in the detachment assay (Section 2.2.3c.), along with similar

dilutions of DMSO alone as a control. Background lysis and detachment was controlled for, with triplicate wells of MEM alone.

Results:- Organic CSC caused increasing detachment with increasing concentrations of CSC, but did not significantly change cell lysis. Furthermore, DMSO alone did not increase either detachment or lysis above control values (Figure 3.6)

Figure 3.7 shows the results of four separate experiments where A549 epithelial cells were exposed to organic CSC. The levels of cell detachment and lysis were essentially the same in each experiment.

3.3.2. Effect of Aqueous CSC on A549 Epithelial Cell Detachment

(a) A Comparison of Aqueous CSC

The most common method for the production of CSC involves bubbling cigarette smoke through fluid contained in a syringe (Holden et al, 1989). This method was compared to a method of production using the tonometer and smoking machine (Section 2.2.5b.).

The two differing methods initially used to make the aqueous CSC's are described in Section 2. These CSC.'s were made fresh on the morning of each experiment, and diluted in MEM to the required concentrations. The aqueous solvent of HBSS was also diluted to give the appropriate control solutions.

Protocol:- The CSC's produced by the tonometer and syringe methods (Section 2.2.5b.) were diluted in MEM to give concentrations of 1,5,10 and 20% and incubated with A549 epithelial cells in the detachment assay for 4 hours at 37°C, 5% CO₂, then harvested.

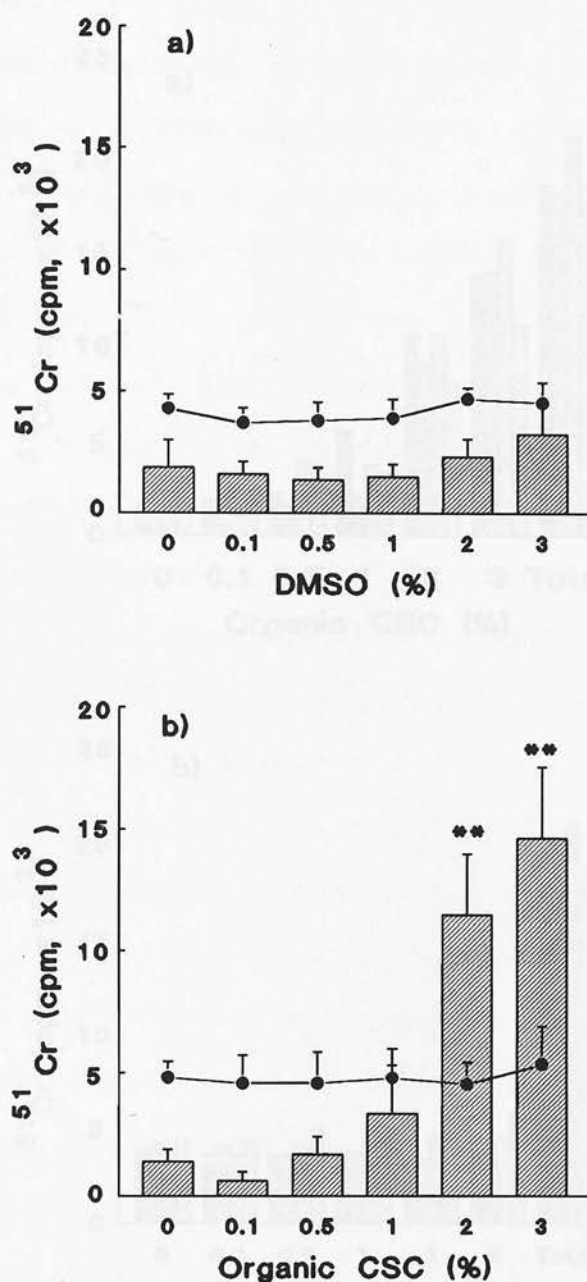


Figure 3.6. The effect of increasing concentrations of a) DMSO, and b) Organic CSC on the detachment (histograms) and lysis (\bullet) of A549 epithelial cells measured in ^{51}Cr cpm. The histograms/ \bullet represent the mean of triplicate wells in four separate experiments and the bars indicate 1 SEM in each case. ** $p < 0.01$ compared with medium alone (\circ).

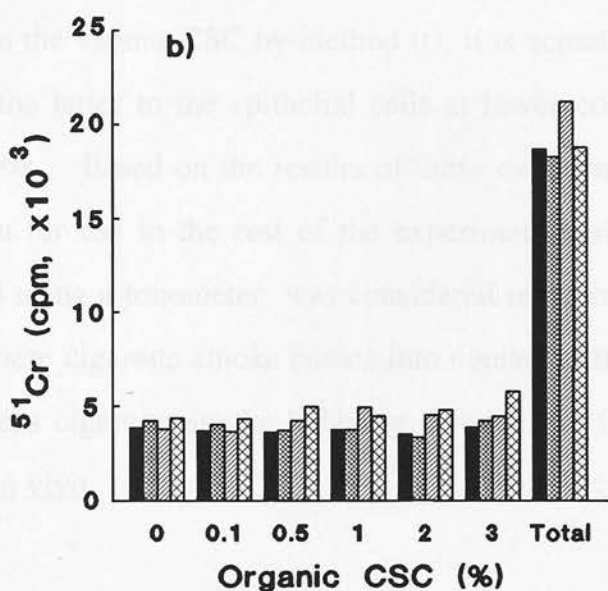
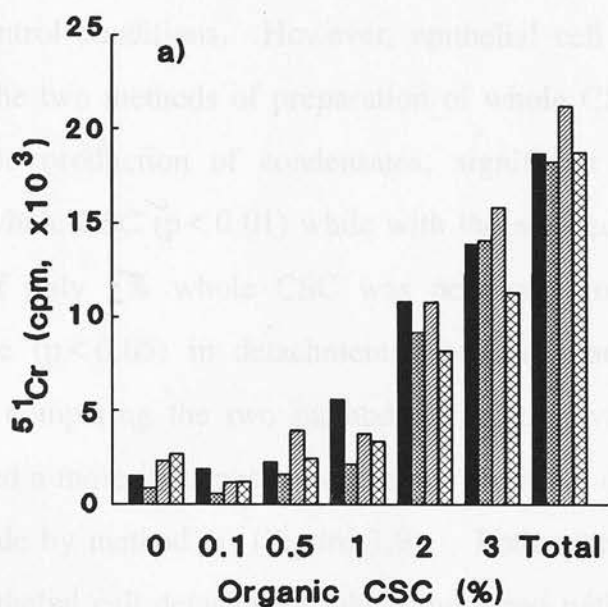


Figure 3.7. The effect of increasing concentration of organic CSC on a) detachment, and b) lysis of A549 epithelial cells in four individual experiments, measured in ^{51}Cr cpm.

Results:- Whole CSC produced by either the syringe (s) or tonometer (t) did not enhance epithelial cell lysis at any of the concentrations used when compared with control conditions. However, epithelial cell detachment differed between the two methods of preparation of whole CSC. Using method (s) for the production of condensates, significant detachment occurred at 50% whole CSC ($p < 0.01$) while with the adapted method (t) a concentration of only 5% whole CSC was necessary to produce a significant increase ($p < 0.05$) in detachment over background (Figure 3.8). Similarly, comparing the two methods of making vapour CSC, method (t) produced a more injurious condensate at the same dilution than the condensate made by method (s) (Figure 3.9). Both types of vapour CSC produced epithelial cell detachment which increased with increasing dose. Although vapour CSC produced by method (s) gives higher values for detachment than the vapour CSC by method (t), it is actually less toxic as the addition of the latter to the epithelial cells at lower concentrations also lead to cell lysis. Based on the results of these experiments, the (t) method was chosen for use in the rest of the experiments using aqueous CSC. This method using a tonometer was considered more relevant to in vivo conditions, where cigarette smoke comes into contact with a thin film of the ELF, whereas cigarette smoke bubbling through the fluid is very unlikely to occur in vivo.

(b) The pH of Aqueous CSC

Although the pH indicator phenol red which was present in the assay MEM showed no gross changes in colour with the addition of the condensates, there could have been smaller changes which were not easily detectable by eye.

For this reason the pH of a range of concentrations of both whole and

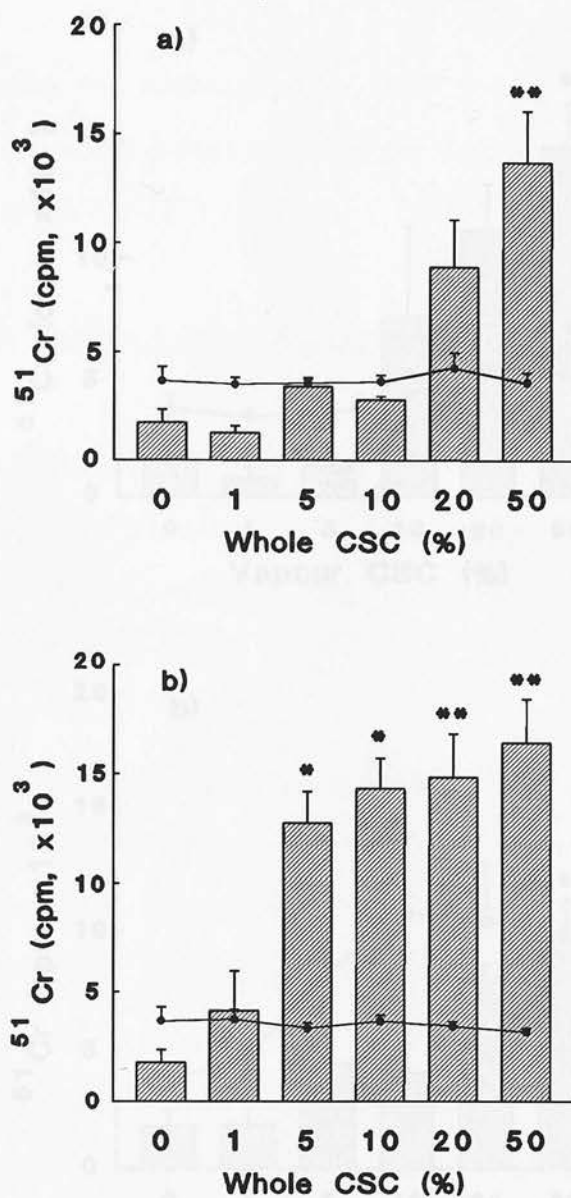


Figure 3.8. The effect of increasing concentrations of whole CSC prepared by a) method s, and b) method t on detachment (histograms) and lysis (\bullet), measured in ^{51}Cr cpm. The histograms/ \bullet represent the mean of triplicate wells in three separate experiments and the bars indicate 1 SEM in each case. * p < 0.05; ** p < 0.01 compared with medium alone (0).

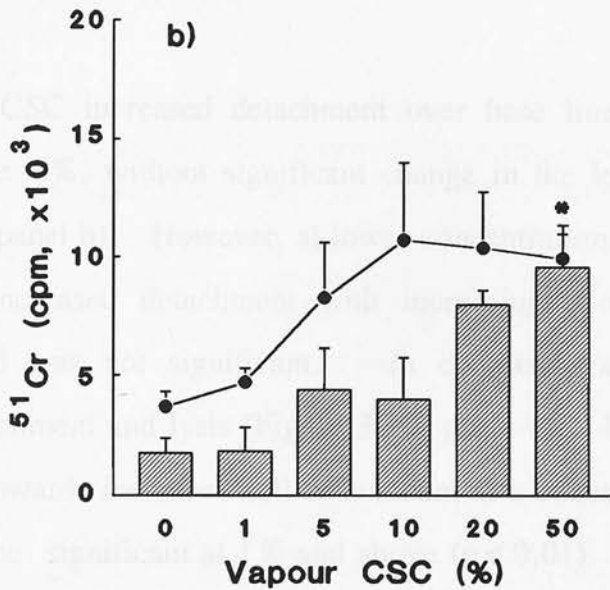
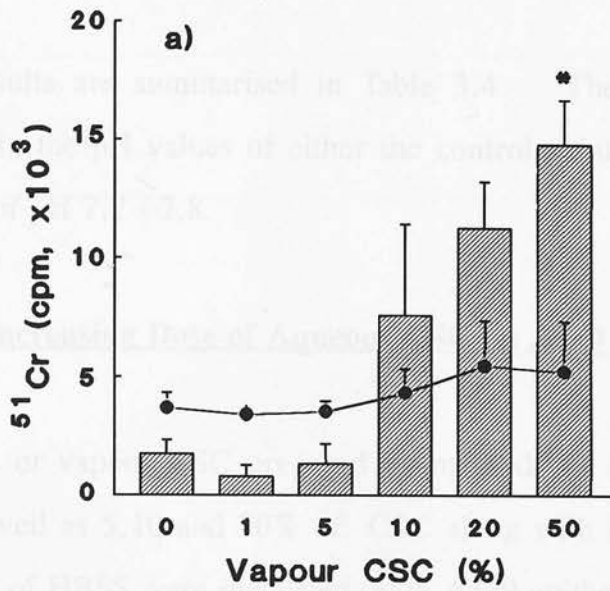


Figure 3.9. The effect of increasing concentrations of vapour CSC prepared by a) method s, and b) method t on detachment (histograms) and lysis (●), measured in ⁵¹Cr cpm. The histograms/● represent the mean of triplicate wells in three separate experiments and the bars indicate 1 SEM in each case. *p < 0.05 compared with medium alone (0).

vapour CSC (0.1,1,2,5,10 and 20%) as well as that of the control HBSS solution was measured using a pH stick (Gallenkamp).

Results:- The results are summarised in Table 3.4. There was no significant change in the pH values of either the control solutions or the condensates range of pH 7.2 - 7.8.

(c) The Effect of Increasing Dose of Aqueous CSC on A549 Epithelial Cell Detachment

Using either whole or vapour CSC prepared by method (t), dilutions of 0.1, 1 and 2% as well as 5,10 and 20% of CSC along with dilutions of the control solution of HBSS were incubated with A549 epithelial cells in the detachment assay (Section 2.2.3c.).

Results:- Whole CSC increased detachment over base line values at concentrations of $\geq 2\%$, without significant change in the level of cell lysis (Figure 3.10, panel b). However, at lower concentrations there was a trend towards increased detachment with increasing concentration, although this trend was not significant. In contrast, vapour CSC increased both detachment and lysis (Figure 3.10, panel c). In this case there was a trend towards increased cell detachment at a concentration of 0.1% which became significant at 1% and above ($p < 0.01$). Similarly cell lysis only became significant at concentrations of $\geq 2\%$, although again there was a trend towards increasing cell lysis at the lower concentration. There were no significant changes in cell detachment or lysis with HBSS treatment (Figure 3.10, panel a).

Comparing individual experiments for the response of A549 epithelial cells following either whole or vapour CSC, individual experiments

| Exposure (puffs) | Whole | | Vapour | |
|------------------|-------|-----------|--------|-----------|
| 0 | 1.12 | (0.09) | 1.12 | (0.09) |
| sham | 1.15 | (0.17) | 1.15 | (0.17) |
| 1 | 3.89 | (0.62) | 3.95 | (0.59) |
| 2 | 7.29 | (1.50) | 6.50 | (1.84) |
| 4 | 11.17 | (1.38)** | 11.17 | (1.39)** |
| 6 | 16.36 | (1.42)*** | 19.35 | (1.12)*** |

Table 3.1. The effect of increasing exposure of whole blood to either whole or vapour phase cigarette smoke, measured in percentage of CoHb. The data represents a mean (SEM) of triplicate measurements in three separate experiments. ** $p < 0.01$; * $p < 0.001$ compared with the control of sham exposed whole blood samples (Sham = 4 puffs of air).**

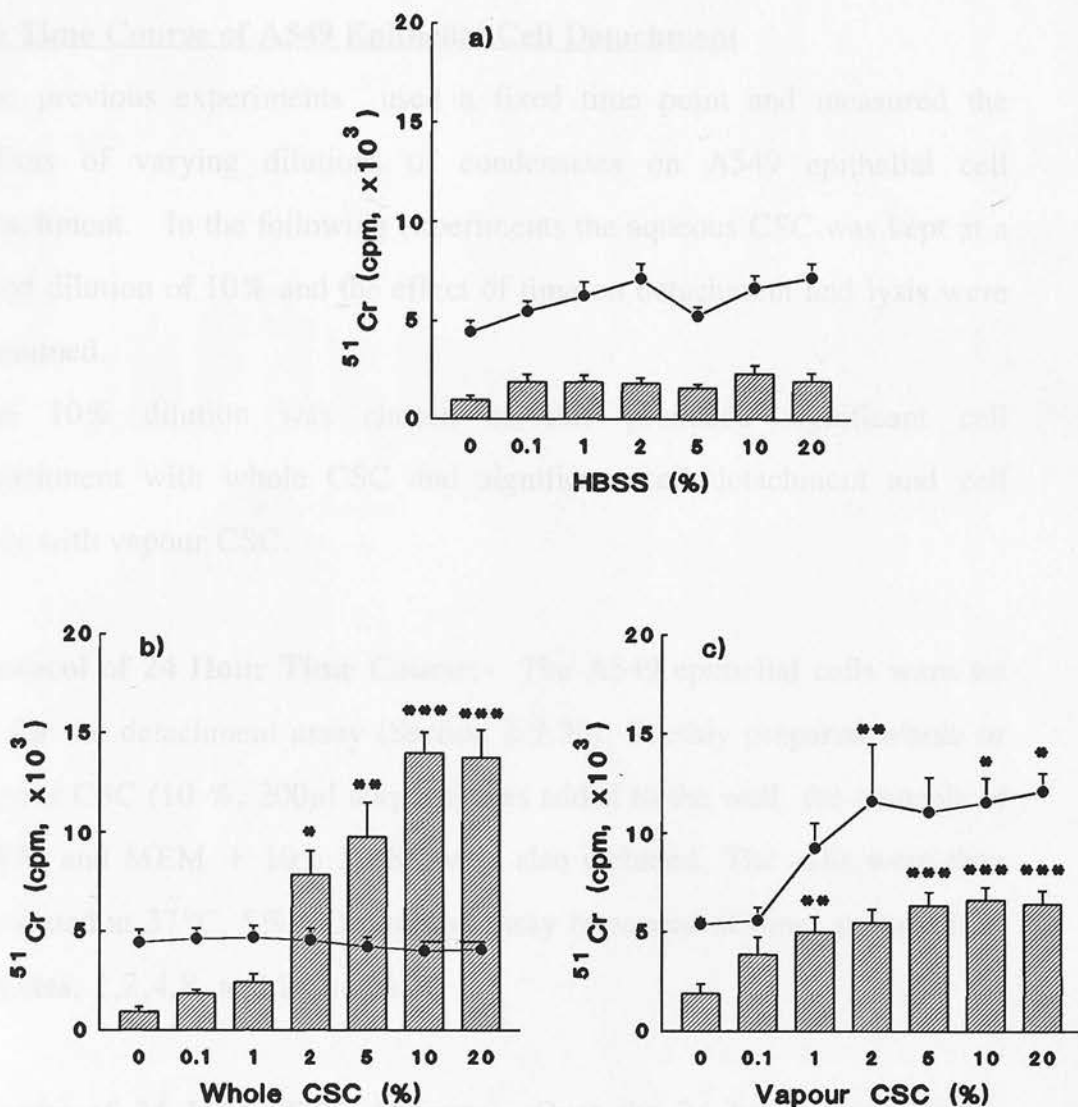


Figure 3.10. The effect of increasing concentrations of a) HBSS, b) whole CSC, and c) vapour CSC on the detachment (histograms) and lysis (●) of A549 epithelial cells measured in ^{51}Cr cpm. The histograms/● represent the mean of triplicate wells in six separate experiments and the bars indicate 1 SEM in each case. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.01$ compared with medium alone (0).

produced the same trend.

(d) Time Course of A549 Epithelial Cell Detachment

The previous experiments used a fixed time point and measured the effects of varying dilutions of condensates on A549 epithelial cell detachment. In the following experiments the aqueous CSC was kept at a fixed dilution of 10% and the effect of time on detachment and lysis were examined.

The 10% dilution was chosen as this produced significant cell detachment with whole CSC and significant cell detachment and cell lysis with vapour CSC.

Protocol of 24 Hour Time Course:- The A549 epithelial cells were set up for the detachment assay (Section 2.2.3b). Freshly prepared whole or vapour CSC (10 %, 200 μ l aliquots) was added to the well, the controls of MEM and MEM + 10% HBSS were also included. The cells were then incubated at 37°C, 5% CO₂ and the assay harvested at times points of 30 minutes, 1,2,4,8, and 24 hours.

Results of 24 Hour Time Course:- Over the 24 hour period of the experiment there was no significant change in the background levels of A549 epithelial cell detachment using either MEM or 10% HBSS in MEM ($p > 0.05$). There was a minimal increase in the extent of cell lysis in both cases at 24 hours. However, this was probably an artifact of the assay since lysis is measured as non-cell associated ⁵¹Cr label c.p.m. in the supernatants and this label is not tightly bound to the cell since it slowly disassociates over 24 hours (Figure 3.11, panel a & b). The addition of whole CSC leads to a gradual increase in epithelial

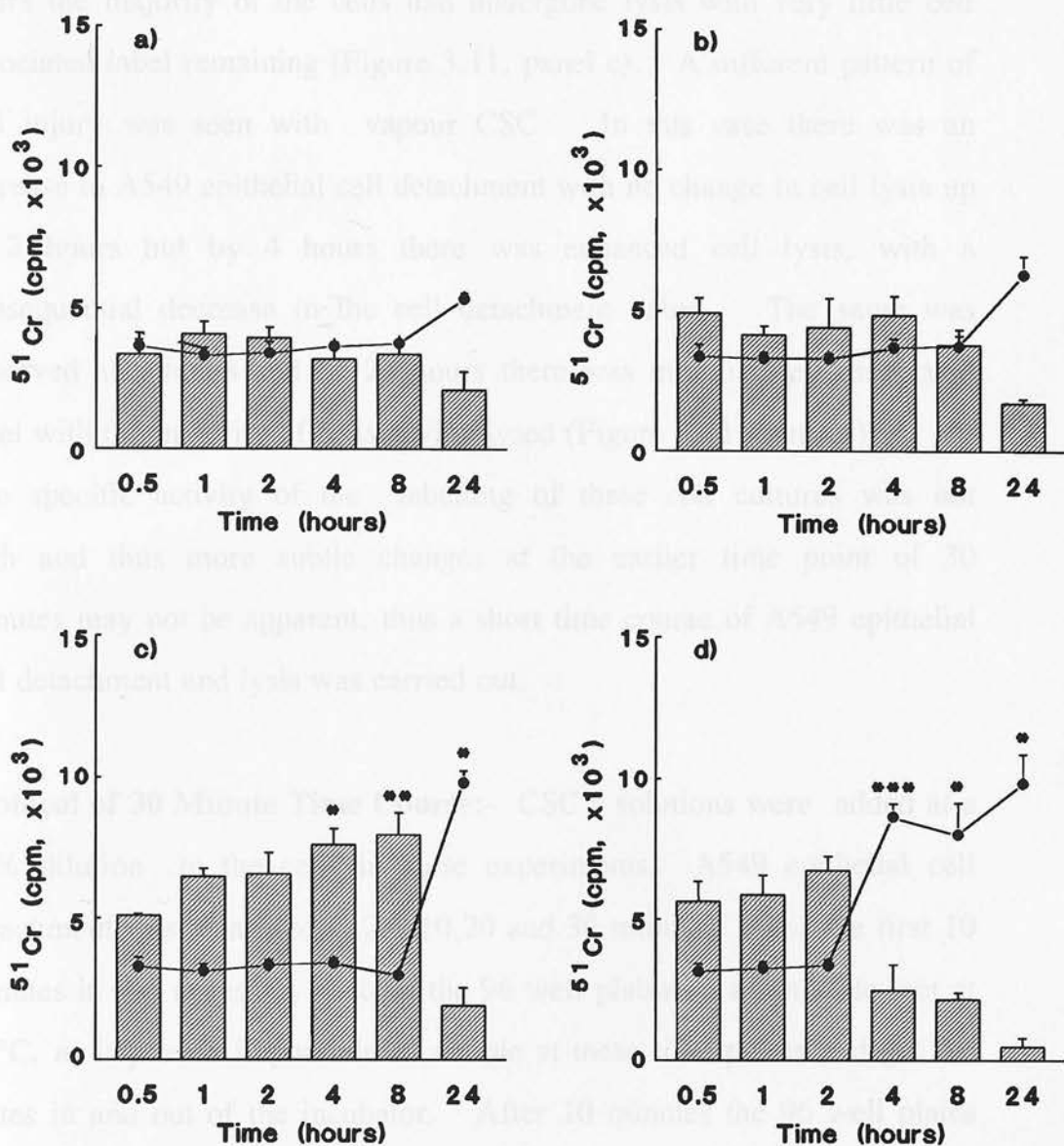


Figure 3.11. The effect of a) MEM, b) 10% HBSS, c) 10% whole CSC, and d) 10% vapour CSC on the detachment (histograms) and lysis (\bullet) of A549 epithelial cells over 24 hours, measured in ^{51}Cr cpm. The histograms/ \bullet represent the mean of triplicate wells in three separate experiments and the bars indicate 1 SEM in each case. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared with medium alone.

detachment up to 8 hours, with no change in cell lysis. However, by 24 hours the majority of the cells had undergone lysis with very little cell associated label remaining (Figure 3.11, panel c). A different pattern of cell injury was seen with vapour CSC. In this case there was an increase in A549 epithelial cell detachment with no change in cell lysis up to 2 hours but by 4 hours there was enhanced cell lysis, with a consequential decrease in the cell detachment value. The same was observed at 8 hours and by 24 hours there was minimal cell associated label with the majority of cells having lysed (Figure 3.11, panel d).

The specific activity of the labelling of these cell cultures was not high and thus more subtle changes at the earlier time point of 30 minutes may not be apparent, thus a short time course of A549 epithelial cell detachment and lysis was carried out.

Protocol of 30 Minute Time Course:- CSC's solutions were added at a 10% dilution to the cells in these experiments. A549 epithelial cell detachment was measured at 2,5,10,20 and 30 minutes. For the first 10 minutes it was necessary to keep the 96 well plates on a hot plate set at 37°C, as it proved impossible to sample at these time points and get the plates in and out of the incubator. After 10 minutes the 96 well plates were incubated between assay time points.

Results of 30 Minute Time Course:- The background detachment for MEM alone or 10% HBSS in MEM was low and constant over the 30 minute period (Figure 3.12, panel a & b). Enhanced A549 epithelial cell detachment became apparent at 30 minutes with both whole and vapour CSC although it was significant ($p < 0.05$) only with whole CSC treatment; no change in cell lysis was observed (Figure 3.12, panel c & d).

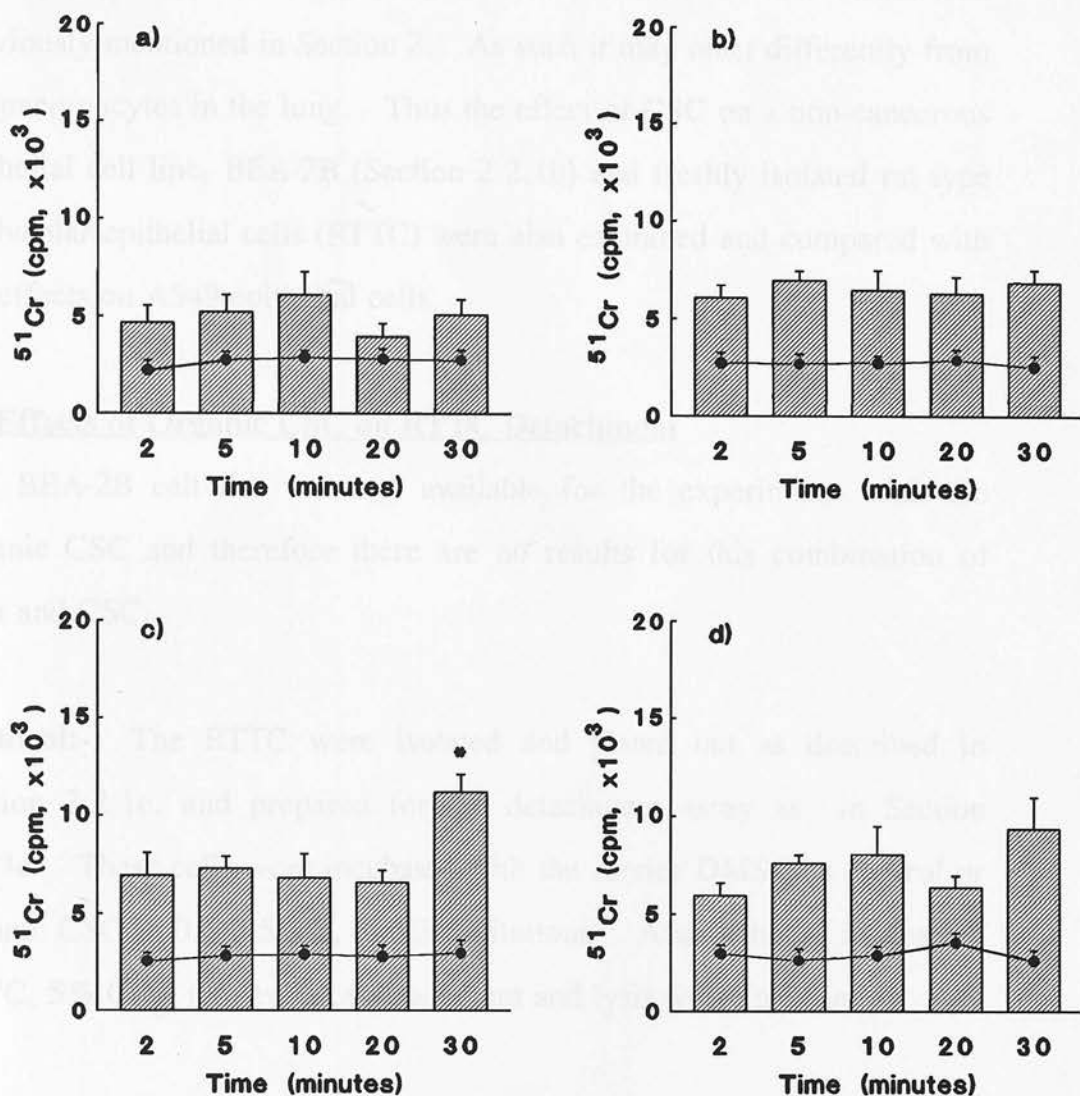


Figure 3.12. The effect of a) MEM, b) 10% HBSS, c) 10% whole CSC, and d) 10% vapour CSC on the detachment (histograms) and lysis (\bullet) of A549 epithelial cells over 30 minutes, measured in ^{51}Cr cpm. The histograms/ \bullet represent the mean of triplicate wells in four separate experiments and the bars indicate 1 SEM in each case. * $p < 0.05$ compared with medium alone.

3.3.3. Comparison of Cell Types

The A549 epithelial cell line is derived from an alveolar cell carcinoma as previously mentioned in Section 2. As such it may react differently from the pneumocytes in the lung. Thus the effect of CSC on a non-cancerous epithelial cell line, BEA-2B (Section 2.2.1b) and freshly isolated rat type II alveolar epithelial cells (RTTC) were also examined and compared with the effects on A549 epithelial cells.

(a) Effects of Organic CSC on RTTC Detachment

The BEA-2B cell line was not available for the experiments with the organic CSC and therefore there are no results for this combination of cells and CSC.

Protocol:- The RTTC were isolated and plated out as described in Section 2.2.1c. and prepared for the detachment assay as in Section 2.2.3c. These cells were incubated with the carrier DMSO as control or organic CSC in 0.1, 0.5, 1, 2, and 3% dilutions. After 4 hours incubation (37°C, 5% CO₂) the levels of detachment and lysis were measured.

Results:- DMSO alone had little effect on either RTTC detachment or lysis compared with control cells (Figure 3.13, panel a). However, organic CSC, although producing minimal cell detachment, did lead to a dose dependent increase in cell lysis which leveled out at 2% (Figure 3.13, panel b). This change in cell lysis was significant at 2% dilution ($p < 0.01$). The uptake of ⁵¹Cr label by the RTTC was less than that of A549 epithelial cells and so caution should be used in comparing the sets of results. However, cultures of A549 epithelial cells did not undergo cell lysis with organic CSC. This suggests that the RTTC were more

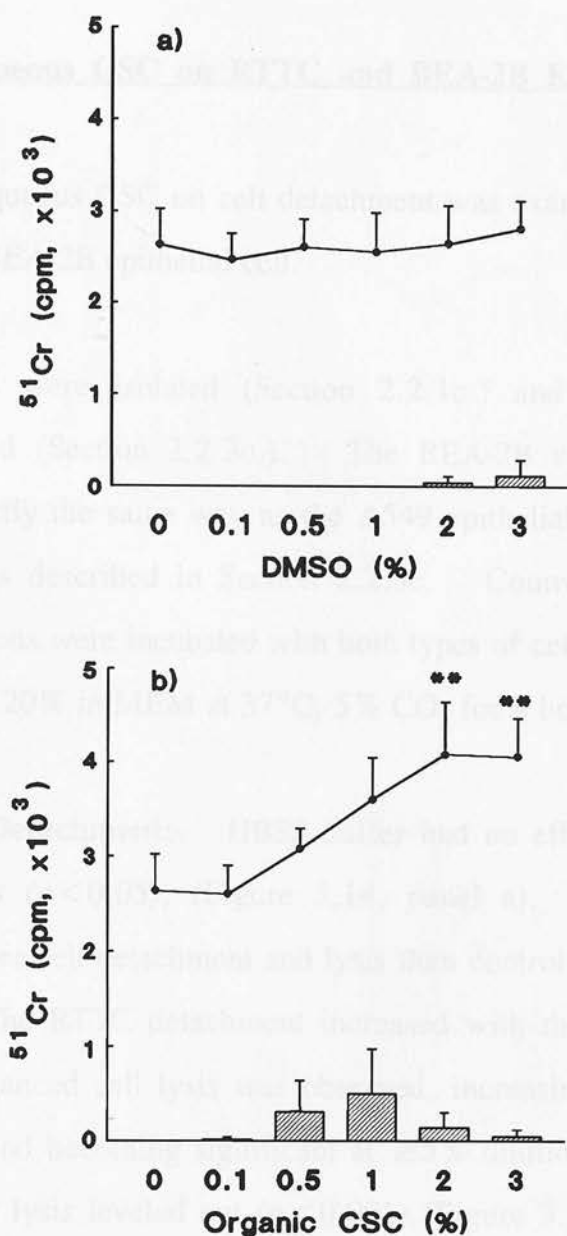


Figure 3.13. The effect of increasing concentrations of a) DMSO, and b) Organic CSC on the detachment (histograms) and lysis (\bullet) of RTTC measured in ^{51}Cr cpm. The histograms/ \bullet represent the mean of triplicate wells in five separate experiments and the bars indicate 1 SEM in each case. ** $p < 0.01$ compared with medium alone (0).

sensitive to the effects of organic CSC than A549 epithelial cells.

(b) Effects of Aqueous CSC on RTTC and BEA-2B Epithelial Cell Detachment

The effect of the aqueous CSC on cell detachment was examined on both the RTTC and the BEA-2B epithelial cell.

Protocol:- RTTC were isolated (Section 2.2.1c.) and prepared as previously described (Section 2.2.3c.). The BEA-2B epithelial cells were set up in exactly the same way as the A549 epithelial cells for the detachment assay as described in Section 2.2.3c. Control (HBSS) or aqueous CSC solutions were incubated with both types of cells at dilutions of 0.1, 1, 2, 5, 10 and 20% in MEM at 37°C, 5% CO₂ for 4 hours.

Results of RTTC Detachment:- HBSS buffer had no effect on RTTC detachment or lysis ($p < 0.05$), (Figure 3.14, panel a). Whole CSC produced both greater cell detachment and lysis than control cells (Figure 3.14, panel b). The RTTC detachment increased with the addition of vapour CSC. Enhanced cell lysis was observed, increasing in a dose-dependent manner and becoming significant at $\geq 5\%$ dilutions of vapour CSC at which point lysis leveled out ($p < 0.001$) (Figure 3.14, panel c). Again as the uptake of ⁵¹Cr label by the RTTC is lower than with A549 cells, care should be taken in making direct comparisons with the effect of the aqueous CSC on A549 epithelial cell detachment. However, the RTTC seem to be more susceptible to the effects of condensate.

Results of BEA-2B Epithelial Cell Detachment:- The HBSS buffer did not produce an increase in either detachment or lysis above background

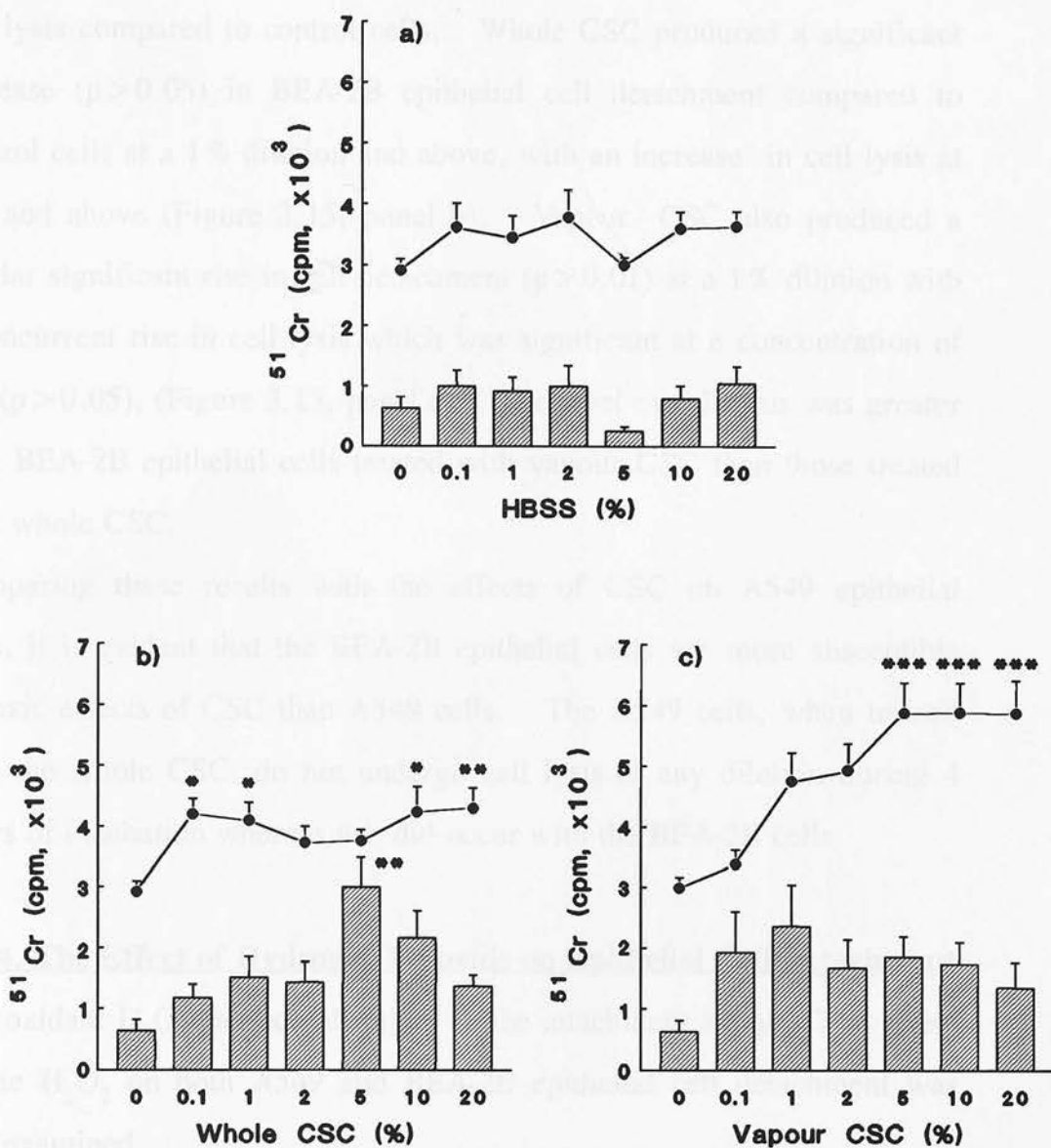


Figure 3.14. The effect of increasing concentrations of a) HBSS, b) whole CSC, and c) vapour CSC on the detachment (histograms) and lysis (—●—) of RTTCs, measured in ^{51}Cr cpm. The histograms/—●— represent the area of triplicate wells in four separate experiments and the bars indicate 1 SEM in each case. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ when compared with medium alone (0).

levels in BEA-2B epithelial cell cultures. Both whole and vapour CSC induced a dose-dependent cell injury with increased cell detachment and cell lysis compared to control cells. Whole CSC produced a significant increase ($p > 0.05$) in BEA-2B epithelial cell detachment compared to control cells at a 1% dilution and above, with an increase in cell lysis at 2% and above (Figure 3.15, panel b). Vapour CSC also produced a similar significant rise in cell detachment ($p > 0.01$) at a 1% dilution with a concurrent rise in cell lysis which was significant at a concentration of 2% ($p > 0.05$), (Figure 3.15, panel c). The level of cell lysis was greater with BEA-2B epithelial cells treated with vapour CSC than those treated with whole CSC.

Comparing these results with the effects of CSC on A549 epithelial cells, it is evident that the BEA-2B epithelial cells are more susceptible to toxic effects of CSC than A549 cells. The A549 cells, when treated with the whole CSC, do not undergo cell lysis at any dilution during 4 hours of incubation whereas this did occur with the BEA-2B cells.

3.3.4. The Effect of Hydrogen Peroxide on Epithelial Cell Detachment

The oxidant H_2O_2 caused cell injury in the attachment assay. The effect of the H_2O_2 on both A549 and BEA-2B epithelial cell detachment was also examined.

Protocol:- Freshly prepared H_2O_2 was added to the A549 and BEA-2B epithelial cell in the detachment assay at concentrations of 5,10,15,20 & $50\mu M$ and incubated at $37^\circ C$, 5% CO_2 for 4 hours, after which cell detachment and cell lysis were measured.

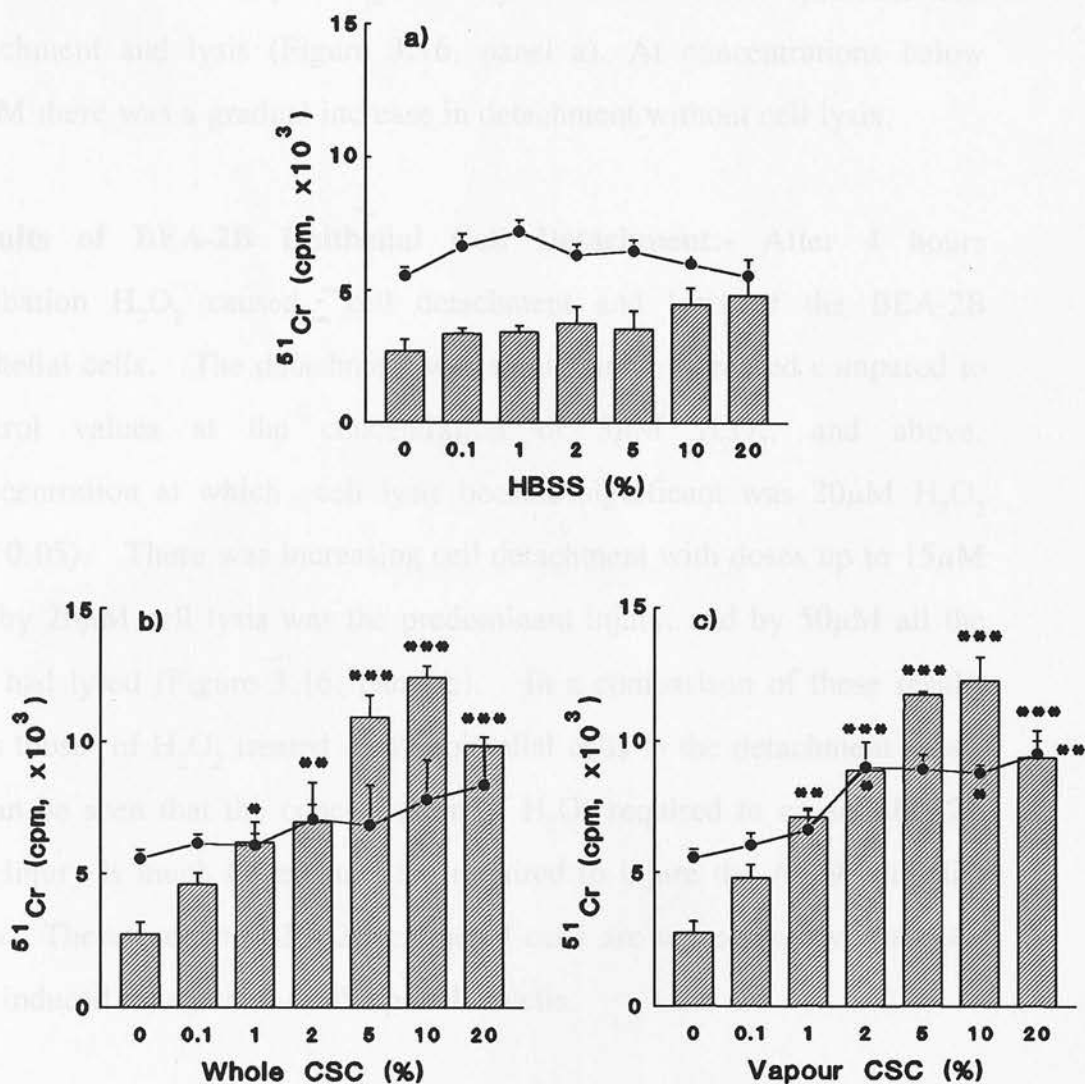


Figure 3.15. The effect of increasing concentration of a) HBSS, b) whole CSC, and c) vapour CSC on the detachment (histograms) and lysis (\bullet) of BEA-2B epithelial cells, measured in ^{51}Cr cpm. The histograms/ \bullet represent the mean of triplicate wells in three separate experiments and the bars indicate 1 SEM in each case. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared with medium alone (0).

Results of A549 Epithelial Cell Detachment:- Hydrogen peroxide at concentration of $\geq 20\mu\text{M}$ significantly increased A549 epithelial cell detachment and lysis (Figure 3.16, panel a). At concentrations below $20\mu\text{M}$ there was a gradual increase in detachment without cell lysis.

Results of BEA-2B Epithelial Cell Detachment:- After 4 hours incubation H_2O_2 caused cell detachment and lysis of the BEA-2B epithelial cells. The detachment was significantly increased compared to control values at the concentration of $5\mu\text{M}$ H_2O_2 , and above. Concentration at which cell lysis became significant was $20\mu\text{M}$ H_2O_2 ($p < 0.05$). There was increasing cell detachment with doses up to $15\mu\text{M}$ but by $20\mu\text{M}$ cell lysis was the predominant injury, and by $50\mu\text{M}$ all the cell had lysed (Figure 3.16, panel b). In a comparison of these results with those of H_2O_2 treated A549 epithelial cells in the detachment assay, it can be seen that the concentration of H_2O_2 required to cause BEA-2B cell injury is much lower than that required to injure the A549 epithelial cells. Therefore, the BEA-2B epithelial cells are more sensitive to H_2O_2 cell induced injury than A549 epithelial cells.

3.4. A549 EPITHELIAL CELL PROLIFERATION

The effects of CSC on A549 epithelial cell proliferation was examined using the $[^3\text{H}]$ thymidine uptake assay. The basis for this assay is that for a cell to multiply it needs to take up and incorporate the components of DNA, one of these being thymidine. Thus cells can be incubated in an exogenous source of $[^3\text{H}]$ labelled thymidine. Ideally if these cells are replicating, the label will be taken up and incorporated into the cells. Realistically other factor, such as repair, will affect this system. Only A549 epithelial cells were used in these experiments.

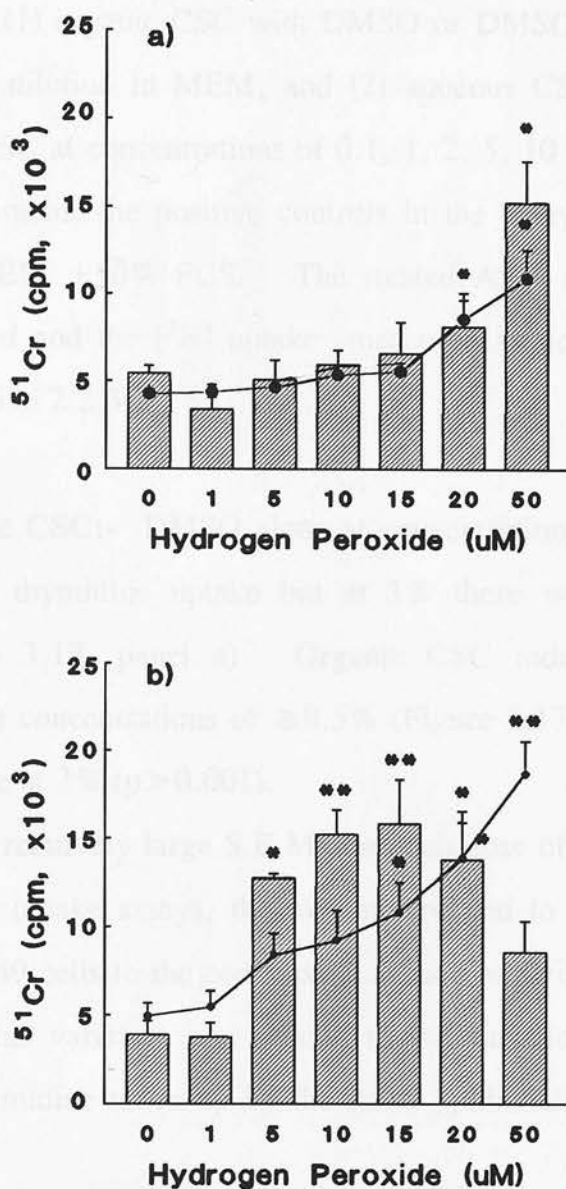


Figure 3.16. The effect of increasing concentrations of H_2O_2 on a) A549, and b) BEA-2B epithelial cell detachment (histograms) and lysis (—●—), measured in ^{51}Cr cpm. The histograms/—●— represent the mean of triplicate wells in three separate experiments for A549 epithelial cells and five separate experiments for BEA-2B epithelial cells, and the bars indicate 1 SEM in each case. * $p < 0.05$; ** $p < 0.01$ compared with medium alone (0).

Protocol of CSC Exposed A549 Epithelial Cells:- The A549 epithelial cells were prepared as described in the first part of Section 2.2.3d. The interventions were (1) organic CSC with DMSO or DMSO alone at 0.1, 0.5, 1, 2 and 3% dilution in MEM, and (2) aqueous CSC with either whole or vapour CSC at concentrations of 0.1, 1, 2, 5, 10 and 20%. In both sets of experiments the positive controls in the assays were MEM +1% FCS and MEM +10% FCS. The treated A549 epithelial cells were then harvested and the [^3H] uptake measured as described in the second part of Section 2.2.3d.

Results of Organic CSC:- DMSO alone at concentrations of 0.1 - 2% did not alter [^3H] thymidine uptake but at 3% there was a decrease ($p < 0.01$), (Figure 3.17, panel a). Organic CSC reduced the [^3H] thymidine uptake at concentrations of $\geq 0.5\%$ (Figure 3.17, panel b) and reached significance at 2% ($p > 0.001$).

Although there are relatively large S.E.M. for each dose of condensate in the [^3H] thymidine uptake assays, this was not related to a variation of response of the A549 cells to the condensate as each experiment followed the same trend. This variation was related to the variation in the total amount of [^3H] thymidine taken up by the A549 epithelial cells between each experiment.

Results of Aqueous CSC:- When exposed to low concentrations (0.1% and 1%) of both types of aqueous CSC there was no change in uptake of [^3H] thymidine when compared with control values. Whole CSC at 2% and 5% produced a fall of approximately 25% in [^3H] thymidine uptake compared with control values, although this fall was not significant. A similar decrease in uptake was also observed in cells

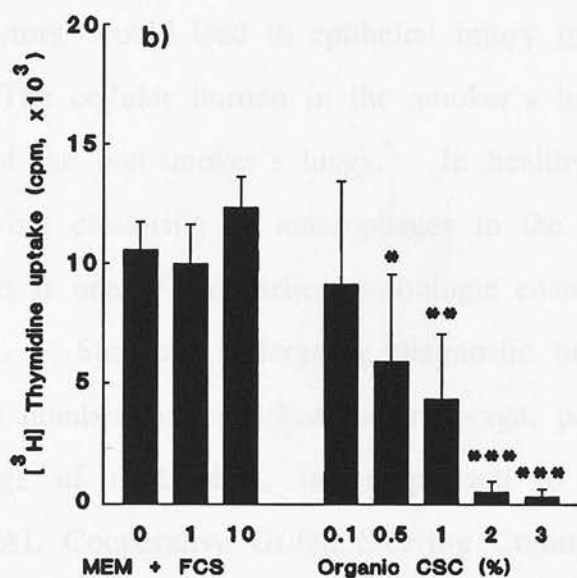
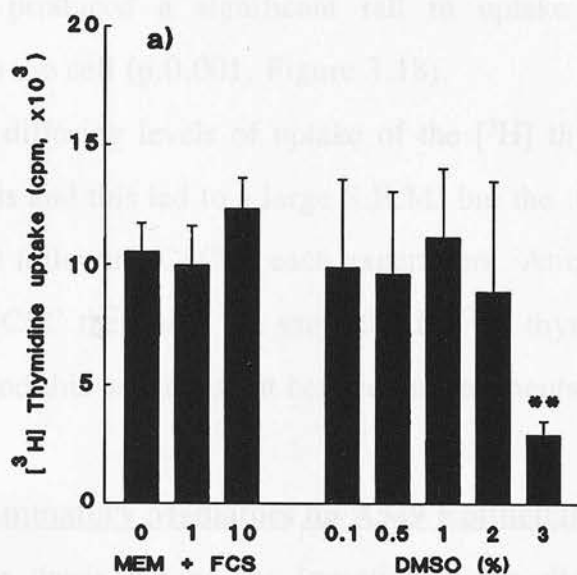


Figure 3.17. The effect of increasing concentrations of a) DMSO, and b) Organic CSC on the [³H] thymidine uptake of A549 epithelial cells measured in [³H] cpm. The histograms represent the mean of triplicate wells in six separate experiments and the bars indicate 1 SEM in each case. *p < 0.05; ** p < 0.01; *** p < 0.001 compared with medium alone (0).

treated with 2% and 5% vapour CSC. However, higher concentrations of CSC ($\geq 10\%$) produced a significant fall in uptake of the [^3H] thymidine label into the cell ($p.0.001$, Figure 3.18).

There were again differing levels of uptake of the [^3H] thymidine label between experiments and this led to a large S.E.M. but the trend towards a decrease occurred following CSC in each experiment. At concentrations of $\geq 10\%$ aqueous CSC there was the same fall in [^3H] thymidine uptake to the same level, and this was constant between experiments.

3.5. Effect of Inflammatory Mediators on A549 Epithelial Cells

This section of the thesis has so far investigated the direct effect of cigarette smoke and CSC's on epithelial injury *in vitro*.

However, other factors could lead to epithelial injury *in vivo* in the smoker's lung. The cellular burden in the smoker's lungs is much greater than that of the non-smoker's lungs. In healthy smokers, a bronchiolitis involving clustering of macrophages in the walls of the terminal bronchioles is one of the earliest pathologic changes observed (Neiwohner, 1982). Smokers undergoing diagnostic bronchoscopy,, have increased cell numbers in bronchoalveolar lavage, particularly an increased percentage of neutrophils, in comparison to non-smokers (Reviewed, The BAL Cooperative Group Steering Committee, 1990). Preliminary experiments were therefore undertaken to examine the effects of inflammatory mediators on A549 epithelial detachment.

Protocol:- The A549 epithelial cells were prepared in the detachment assay as described in Section 2.2.3c. The following three components,, (1) The bacterial endotoxin, Lipopolysaccharide (LPS) at 0.01, 0.1 and $1\mu\text{g.ml}^{-1}$, (2) Tumour Necrosis Factor (TNF) at 50, 100, 200 and 400

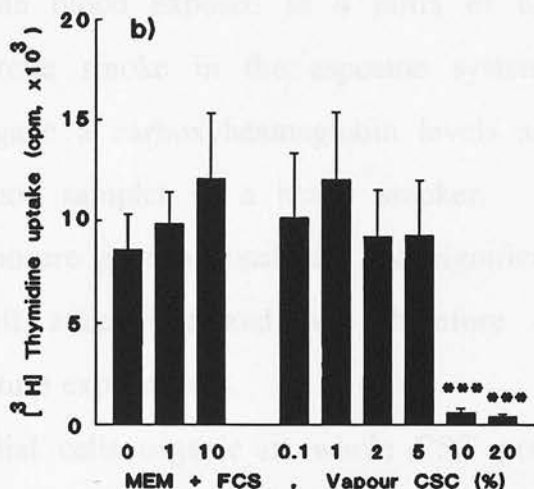
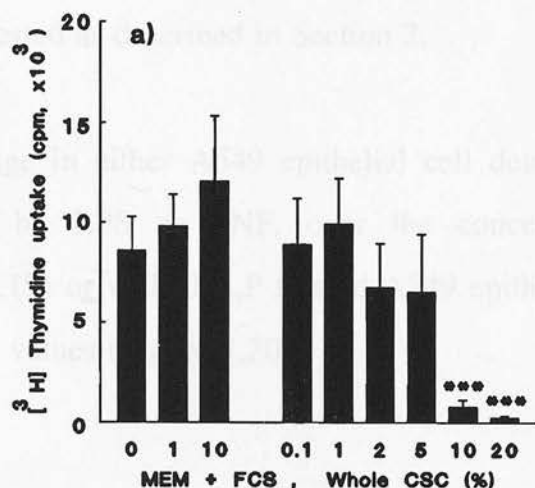


Figure 3.18. The effect of increasing concentrations of a) whole CSC, and b) vapour CSC on the [³H] thymidine uptake of A549 epithelial cells, measured in [³H] cpm. The histograms represent the mean of triplicate wells in six separate experiments and the bars indicate 1 SEM in each case. *** p < 0.001 compared with the medium alone (0).

units ml^{-1} , and (3) (fMLP) at 10, 100 and 1000nM, were dissolved in MEM and added to the detachment assay. The cells were incubated for 4 hours and then harvested as described in Section 2.

Results:- No change in either A549 epithelial cell detachment or cell lysis was induced by LPS or TNF, over the concentration ranges examined (Figure 3.19) or with fMLP treated A549 epithelial cells when compared to control values (Figure 3.20).

SUMMARY

Anticoagulated whole blood exposed to 4 puffs of either whole or vapour phase cigarette smoke in the esposine system described in these experiments gave a carboxyhaemoglobin levels similar to those seen in venous blood samples in a heavy smoker. This level of cigarette smoke exposure gave a consistent and significant decrease in A549 epithelial cell attachment and was therefore chosen as the standard level for future experiments.

Using A549 epithelial cells organic or whole CSC produced a dose-dependent increase cell detachment only, while vapour CSC gave both a dose-dependent increase cell detachment and lysis. However, when the bronchial epithelial cell line BEA-2B or primary culture type II cells were treated with the CSC both organic or whole CSC were also capable of inducing cell lysis. Thus the A549 epithelial cells were more resistant to CSC induced damage. Organic CSC reduced the [^3H] thymidine uptake of A549 epithelial cells but so did the solvent DMSO. Further experiments using organic CSC were limited to a 2% dilution where there was no obvious interference from DMSO and the dilution caused a

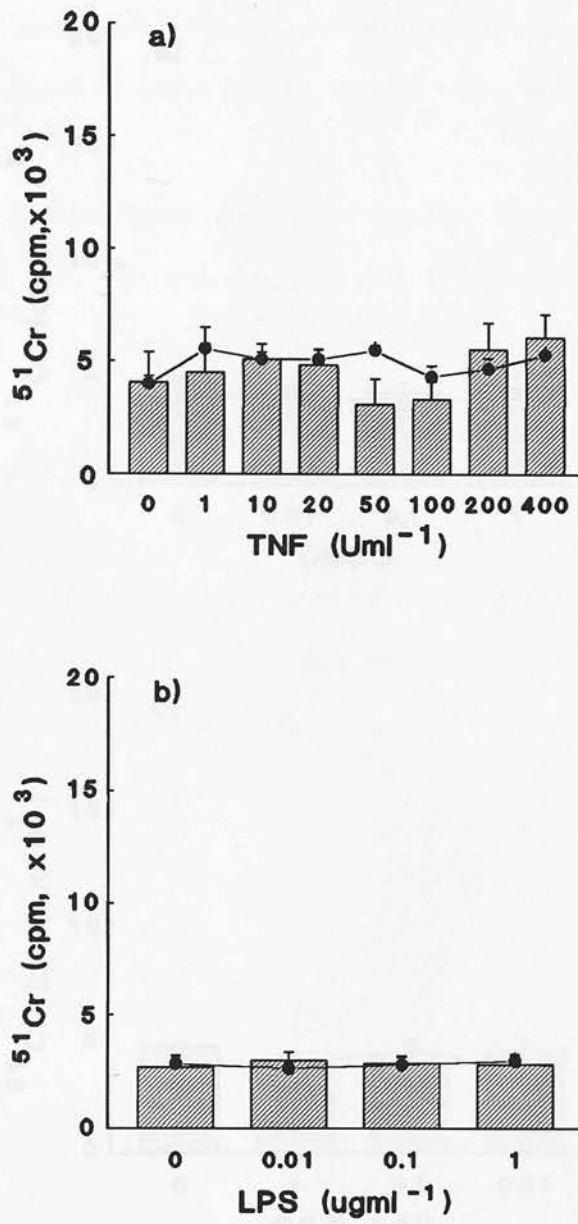


Figure 3.19. The effect of increasing concentrations of a) TNF, and b) LPS on the cell detachment (histograms) and lysis (\bullet) of A549 epithelial cells, measured in ^{51}Cr cpm. The histograms/ \bullet represent the mean of triplicate wells in four separate experiments for TNF and seven separate experiments for LPS, and the bars indicate 1 SEM in each case. There was no significant difference when comparing either TNF or LPS with medium alone (0).

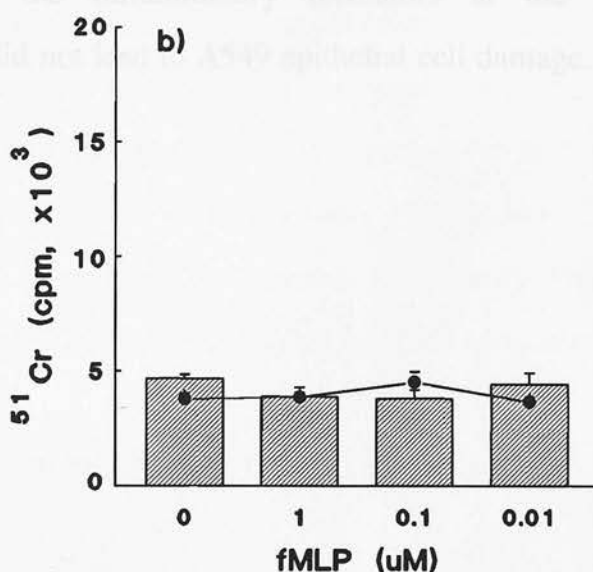
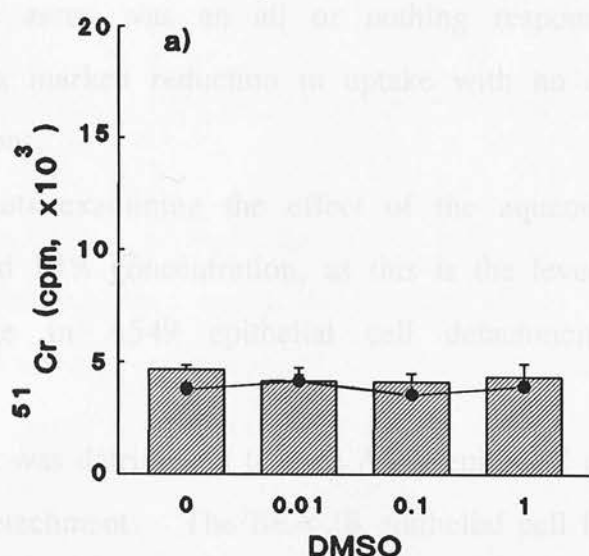


Figure 3.20. The effect of increasing concentrations of a) DMSO and b) fMLP on the detachment (histograms) and lysis (\bullet) of A549 epithelial cell, measured in ^{51}Cr cpm. The histograms/ \bullet represent the mean of triplicate wells in four separate experiments and the bars indicate 1 SEM in each case. There was no significant difference when comparing DMSO/fMLP with medium or fMLP with DMSO. The DMSO units are equivalent units to the fMLP actual concentrations.

constant, significant level of cell injury.

The effect of aqueous CSC on A549 epithelial cells on the [^3H] thymidine uptake assay was an all or nothing response with 10% dilution causing a marked reduction in uptake with no change at the lower concentrations.

Further experiments examining the effect of the aqueous CSC were used at a standard 10% concentration, as this is the level that gives a significant change in A549 epithelial cell detachment, lysis and proliferation.

The oxidant H_2O_2 was detrimental to both A549 epithelial cell producing attachment and detachment. The BEA-2B epithelial cell line was more sensitive to H_2O_2 induced cell damage than the A549 epithelial cells.

The addition of the inflammatory mediators at the concentration ranges examined did not lead to A549 epithelial cell damage.

4.1. NORMAL MORPHOLOGY OF EPITHELIAL CELLS

4.1.1. Visualization by Light Microscopy

A549 and BEA-2B epithelial cells as well as the RTIC were examined to establish their normal appearance under light microscopy.

Protocol for A549 and BEA-2B Epithelial Cells: Both types of epithelial cells were plated out at the low concentration of 1×10^5 cells/ml on to 8 chamber slides and incubated for 24 hours at 37°C. 92

CHAPTER 4

EPITHELIAL CELL MORPHOLOGY

In Chapter 3, the effects of cigarette smoke and its' condensates on epithelial cells were examined within the function assays. The results suggested that cell damage was occurring. This was further investigated by assessing for changes in the appearance of the cells. A549 epithelial cells were used in all the following experiments, with the BEA-2B epithelial cells only being used in selected experiments.

The A549 epithelial cell is thought to behave as a type II epithelial cell and therefore should fit certain criteria (Section 2.2.1a.). The general appearance and structure of these cells was assessed by light microscopy transmission and scanning electron microscopy and freeze fracture.

To verify that the cells used in the primary culture type II pneumocyte were actually type II cells, phase contrast microscopy and transmission electron microscopy (T.E.M.) was carried out on a sample of cells.

4.1. NORMAL MORPHOLOGY OF EPITHELIAL CELLS

4.1.1. Visualisation by Light Microscopy

A549 and BEA-2B epithelial cells as well as the RTTC were examined to establish their normal appearance under light microscopy.

Protocol for A549 and BEA-2B Epithelial Cells:- Both types of epithelial cells were plated out at the low concentration of 5×10^5 cells ml^{-1} on to 8 chamber slides and incubated for 24 hours at 37°C , 5%

CO₂, leaving the cells to adhere and spread (Section 2.2.3e.). The cells were subconfluent at this time, which allowed the cell to spread in any direction. The epithelial cells were washed twice with PBS (400μl) to remove non-adherent cells, then fixed in alcohol and stained with Diff Quik. The cells were visualised using light microscopy at low magnification of x 10 and photographed using a Zeiss MK111 photomicroscope (Germany).

Visualisation:- The A549 epithelial cells were elongated with a pale staining cytoplasm and a single round prominent nucleus with nucleoli also visible (Figure 4.1, panel a). The BEA-2B epithelial cells were also well spread with a frothy pale staining cytoplasm and a single round prominent nucleus with nucleoli visible (Figure 4.2, panel a).

Protocol for RTTC:- RTTC were isolated as described in Section 2.2.1c, then added in well culture plates and incubated at 37°C, 5% CO₂ for 1 day, this allowed the cells to adhere. The cells were then visualised using a reverse-phase photomicroscope (mag. x 10, NIKON, Japan).

Visualisation:- Figure 4.3, panel a, shows a group of RTTC clustered together, which are representative of the majority of cells present in culture. The RTTC are the cells with the nucleus surrounded by numerous dark granule which are the lamellar bodies containing phospholipid.

4.1.2. Visualisation by Electron Microscopy

The two epithelial cell lines and the RTTC were also examined more closely using T.E.M. The A549 epithelial cells were also examined

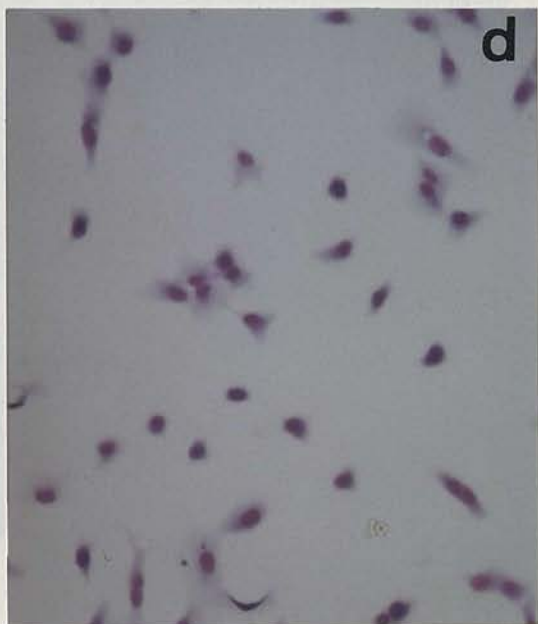
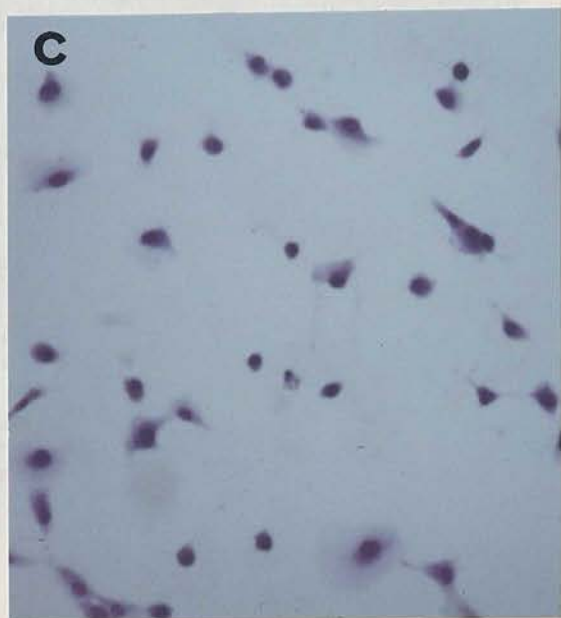
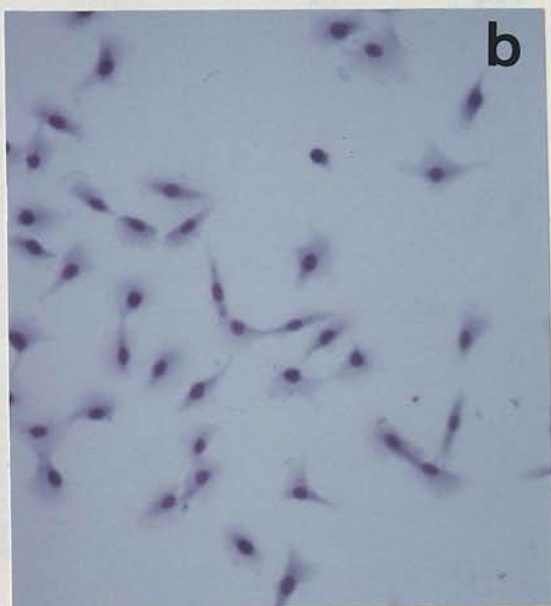


Figure 4.1. Light microscope image of A549 epithelial cells treated with a) MEM, b) 10% HBSS, c) 10% whole CSC, and d) 10% vapour CSC and stained with Diff-Quik to demonstrate morphology. The cells treated with either MEM or 10% HBSS (panels a and b) had a pale staining cytoplasm. The CSC treated cells (panels c and d) had darker, more condensed cytoplasm. (Magnification x 10)

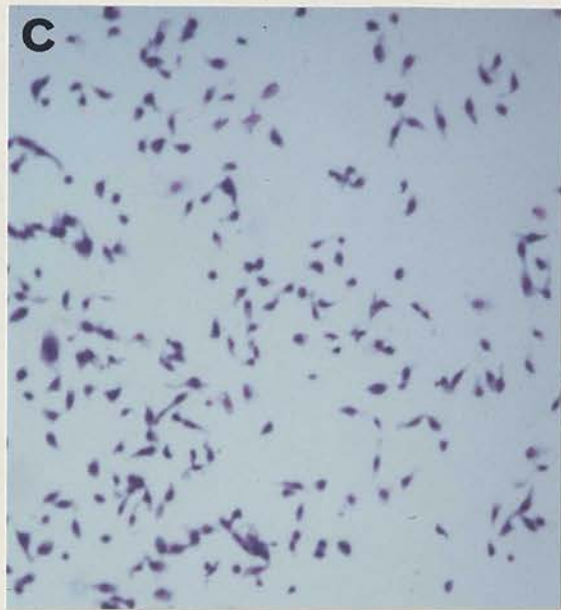
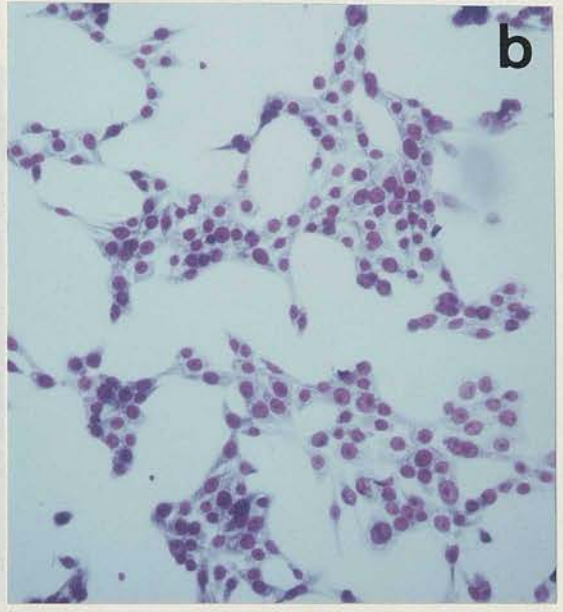
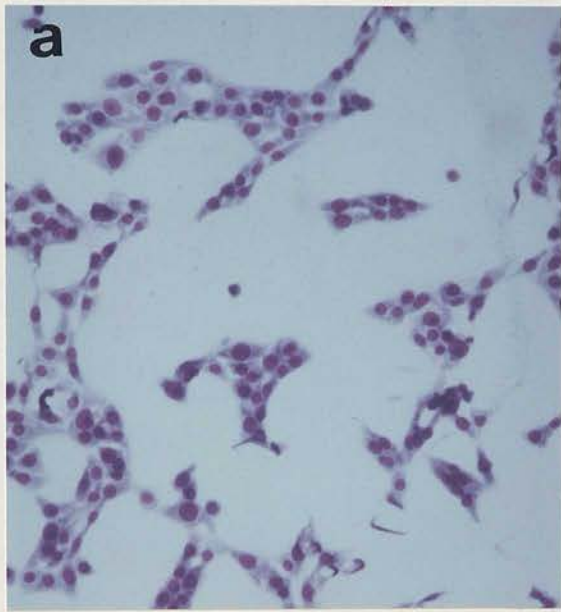


Figure 4.2. Light microscope image of BEA-2B epithelial cells treated with a) MEM, b) 10% HBSS, c) 10% whole CSC, and d) 10% vapour CSC and stained with Diff-Quik to demonstrate morphology. The cells treated with either MEM or 10% HBSS (panel a and b) had a pale staining frothy cytoplasm, with visible nucleoli. The CSC treated cells (panel c and d) were smaller in size with less cytoplasm visible. (Magnification x10)

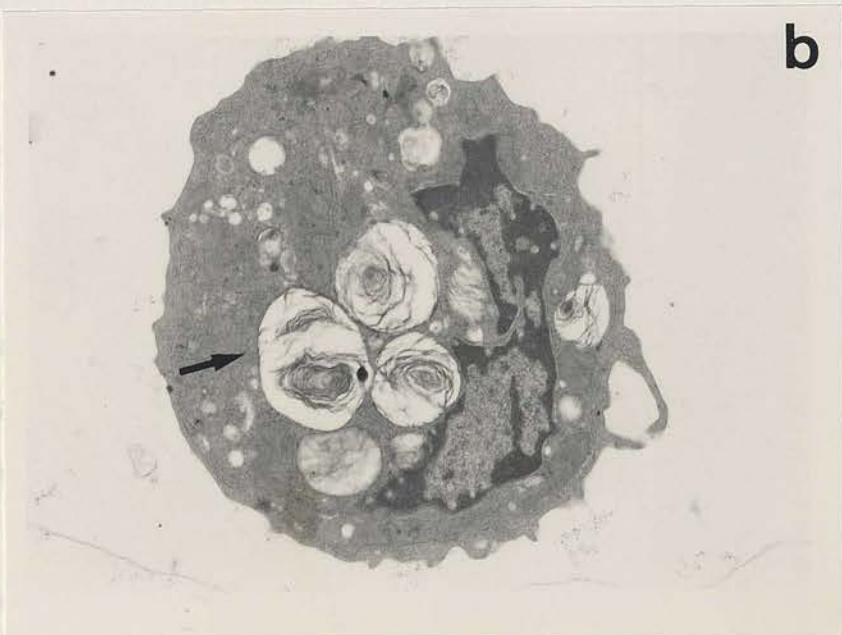
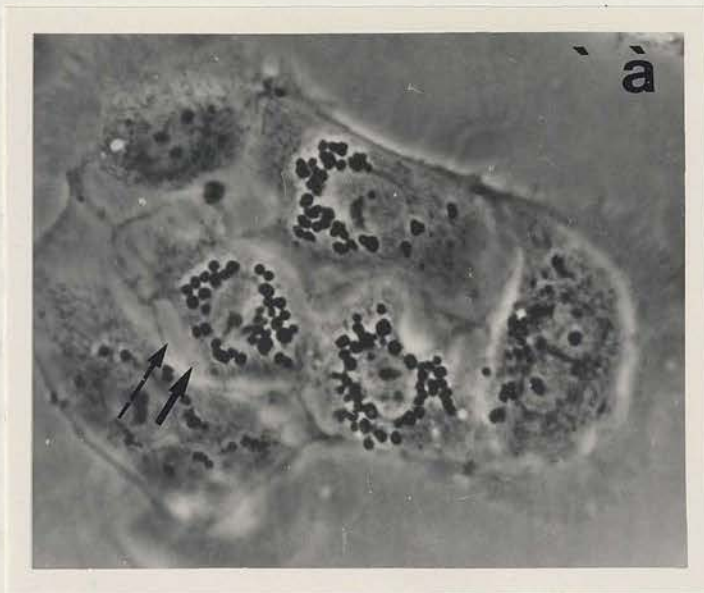


Figure 4.3. Panel a) is a reverse-phase contrast microscope image of RTTC. Panel b) is a T.E.M. image of RTTC. Note the lamellar bodies (arrows) in both panels a) and b). (Magnification a) x10; b) x6k)

using scanning electron microscopy (S.E.M). These cells were processed for T.E.M. by Department of Pathology, University of Edinburgh, and for S.E.M. the Botany Department of the University of Edinburgh. The T.E.M. processing is a standard method with samples post fixation being placed in 1% osmium tetroxide in sodium cacodylate buffer for 1 hour, dehydrated through to absolute alcohol (10,50 and 100%, 15 minute per change) then transferred to propylene oxide (10 minutes, twice) and impregnated overnight in Araldite resin. This preparation was finally embedded in Araldite resin and polymerised at 50°C. The sections were cut on a LKB Ultratome Nova to a thickness of 69 nm and stained with uranyl acetate and lead citrate. These cells were then photographed on a Jeol 100S electron microscope.

The method used for the S.E.M. processing involved dehydration of cells through acetone and critical point drying (Polaron, Fison, UK). These cells were subsequently viewed and photographed on Cambridge S250 stereoscan electron microscope (Leica Cambridge Ltd., Cambridge, UK).

Preparation of Epithelial Cells for T.E.M.:- A549 epithelial cells were trypsinized off the culture flasks as in Section 2.2.1a., resuspended in MEM with 10% FCS and left to recover in teflon wells for 1 hour at 37°C, 5% CO₂. After this the cells were washed twice with PBS (200g, 5 minutes) to remove residual protein, then resuspended in PBS (2 x 10⁶ cells ml⁻¹). To this a 5% gluteraldehyde (electron microscopy grade) - PBS solution was added in a drop-wise fashion to the cell suspension to fix the cells. These cells were then processed for T.E.M. or S.E.M. The BEA-2B were treated in a similar fashion except that these were stripped off the culture flasks as described in Section 2.2.1b. and resuspended in LHC-9 for incubation in the teflon wells and processed for

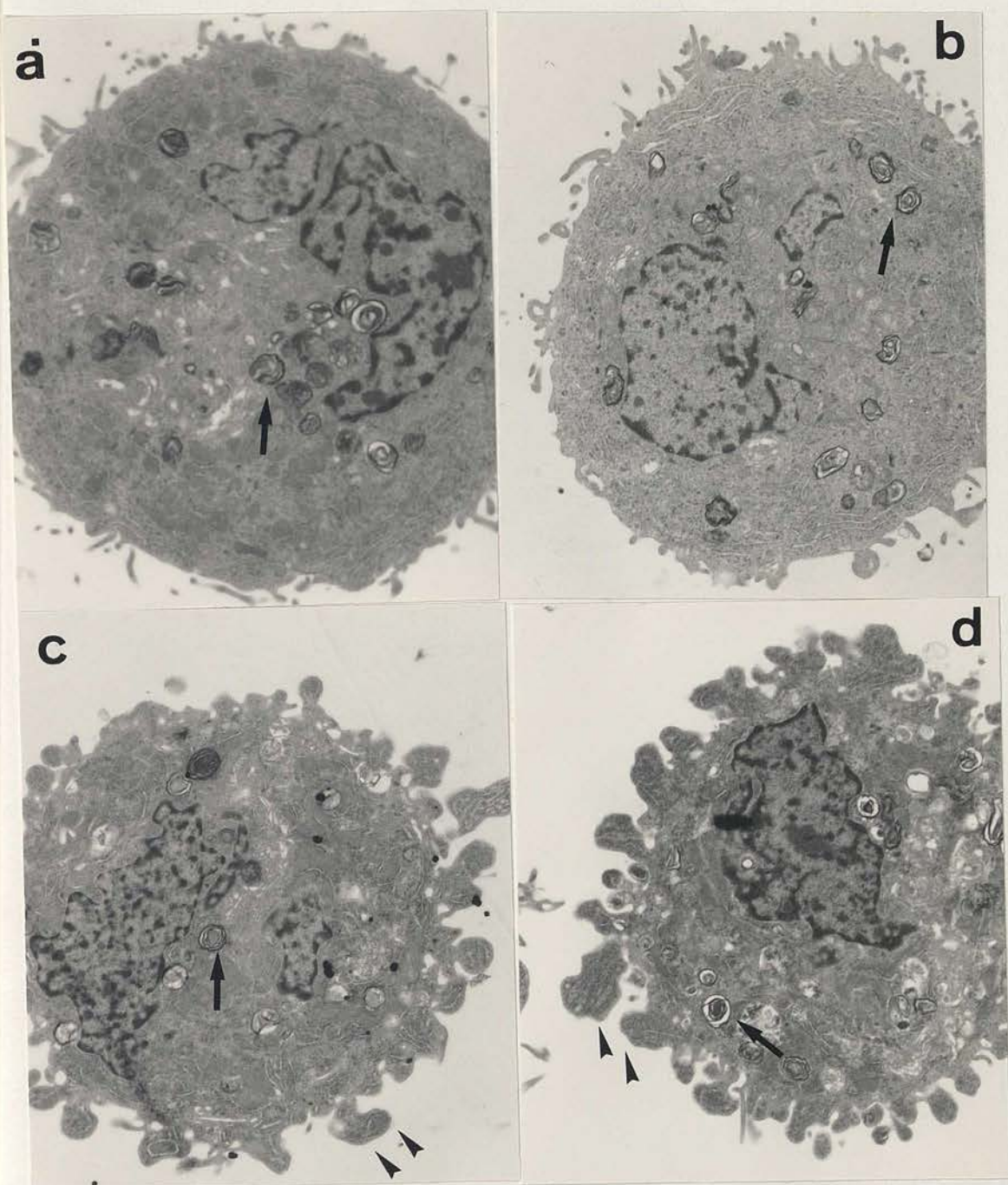
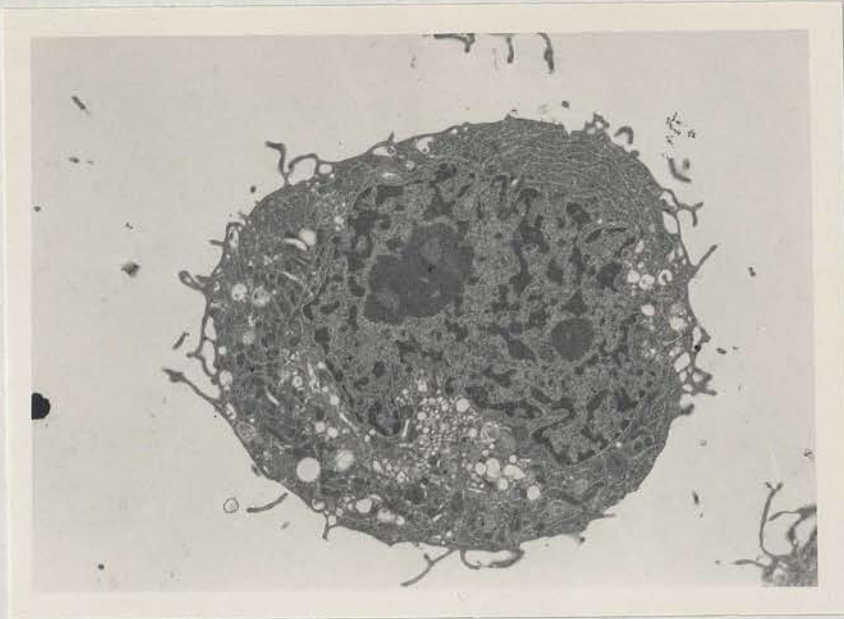


Figure 4.4. T.E.M. image of A549 epithelial cells a) untreated, b) sham exposed, c) 4 puffs of whole cigarette smoke, and d) 4 puffs vapour phase cigarette smoke. Note the lamellar bodies (arrows) in all panels and the surface blebbing (arrowheads) of cells exposed to either whole or vapour phase cigarette smoke (panel c and d). (Magnification x5k)



**Figure 4.5. TEM image of a BEA-2B epithelial cell.
(Magnification x4k)**

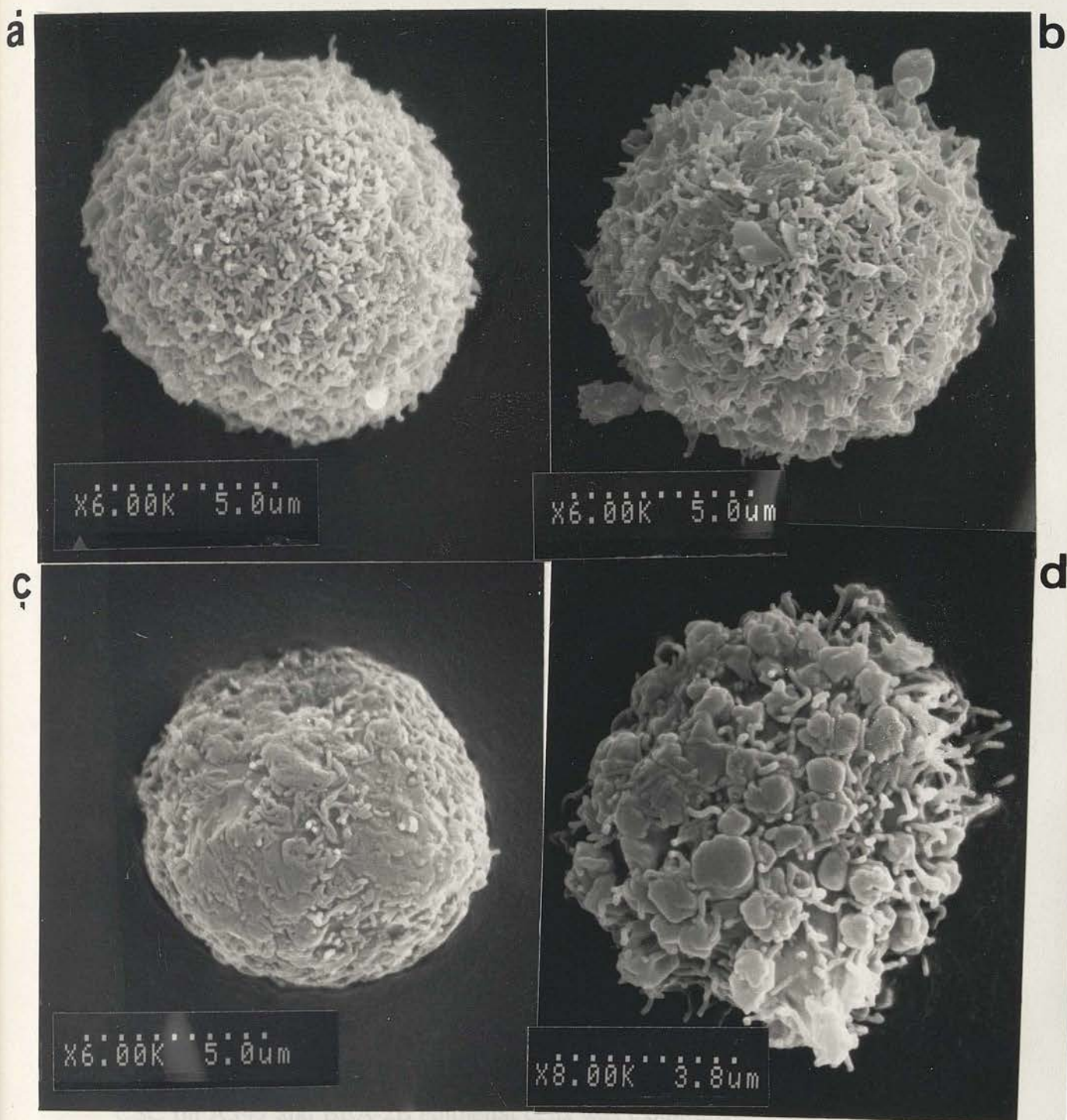


Figure 4.6. S.E.M. image of A549 epithelial cells. a) untreated, b) sham exposed, c) and d) cigarette smoke exposed. Note the ruffled membrane of the control cells (panels a and b). Cigarette smoke exposure led to either the cobbled appearance (panel c) or the blebbing (panel d) of the cell membrane. (Magnification a), b), and c) x6k; d) x8k). The dotted line on each micrograph gives the scale.

culture by another member of the research group, Dr. X.Y. Li, in collaboration with Dr. P.K. Jeffery Research Group (National Heart and Lung Institute, London), by freeze-fracture electron microscopy (E.M.) technique.

Preparation:- Tissue culture inserts (10mm) were added into a 24-well tissue culture plate, each well containing 500 μ l of MEM plus 10% FCS. The inserts were then seeded with A549 epithelial cells (500 μ l, 5×10^5 cells ml⁻¹) and these cells were incubated at 37°C, 5% CO₂ for 1 week. The medium was changed every other day, by which time a monolayer was formed. The inserts were then gently washed with PBS twice and a 2.5% glutaraldehyde solution (0.1M sodium cacodylate buffer at pH 7.2) was added. The cells were then left for 1 hour at 4°C to fix. Thereafter the glutaraldehyde solution was replaced with cacodylate buffer alone and these fixed inserts were then processed for and examined by freeze fracture E.M. using a Philips EM 301 electron microscope (Holland).

Visualisation:- On freeze fracture of monolayers of A549 cells, tight junctions were apparent (Figure 4.7, courtesy of Dr. P.K. Jeffery, National Heart and Lung Institute, London).

Thus the morphology of A549 epithelial cells was considered to be relatively normal, The effect of exposure to either cigarette smoke or CSC on A549 and BEA-2B epithelial cells was examined.

4.2. EFFECTS OF CIGARETTE SMOKE AND CSC ON EPITHELIAL CELL MORPHOLOGY

The effect of cigarette smoke exposure on A549 epithelial cells was examined using suspensions of these cells processed for T.E.M. and

S.E.M. The epithelial cells treated with CSC were visualised by light microscope.

4.2.1. EMT

Protocol
suspension
cigarette m
after wash
cell ml⁻¹)

S.E.M. as before.



Figure 4.7. Electron microscope image of a freeze fracture of an A549 epithelial cell monolayer. Note the tight junctions (arrows) going from the top left hand corner down to the bottom right hand corner.

levels of exposure although this was not always the case. The microvilli present on the membrane of the control cell (Figure 4.6, panel a) had been replaced by membrane blebbing in both cases. This was not an effect of cell agitation in the centrifuge since the CSC treated cells resembled control cells with no effect (Figure 4.6, panel c).

Visualisation by S.E.M.:- The control A549 epithelial cells were spherical with a ruffled cell surface (Figure 4.6, panel a). This was not apparent in the sham-exposed cells (Figure 4.6, panel b). The CSC treated epithelial cells exposed to either whole or fractionated CSC medium kept their general spherical shape but the surface was more irregular. The surface had a cobbled appearance of gaps in the membrane (Figure 4.6, panel c). The cells also showed signs of apoptosis, (Figure 4.6, panel d).

4.2.2. Effect of CSC Exposure on Epithelial Cell Morphology

The effect of CSC's on both epithelial cell lines was examined using light microscopy and S.E.M. It would have been inappropriate to use T.E.M. since this series of experiments studied the effects of condensate on adherent cells, and T.E.M. requires cell suspensions for processing. The experiments were first carried out on A549 epithelial cells and then repeated using the BEA-2B epithelial cells.

Protocol of CSC Exposure for Light Microscopy:- The A549 and BEA-2B epithelial cells were plated out on the 8 chamber glass slides as described in Section 4.1.1. These were then washed twice with PBS (400 μ l, aliquots) to remove non-adherent cells and CSC's added in a 10% dilution in MEM, along with a control solution of 10% HBSS in MEM. After incubating the cells for 4 hours at 37°C, 5% CO₂, the slides were again washed twice with PBS (400 μ l), to remove non-adherent cells fixed and stained with Diff Quik. These were examined under light microscopy (mag. x 10) and photographed on the Zeiss MK111 photomicroscopy.

Visualisation of A549 Epithelial Cells:- A549 epithelial cells treated with either whole or vapour CSC were similar in appearance (whole: Figure 4.1, panel c, and vapour: Figure 4.1, panel d). The cytoplasm stained darker, and was in smaller area than the untreated cells. The nucleus staining varied, some were normal whilst the majority were more condensed with darker staining.

Visualisation of BEA-2B Epithelial Cells:- Cells treated with the

control solutions of MEM were well spread with a pale frothy staining cytoplasm and a darker staining nucleus. This was also the case for cells treated with the control solution of 10% HBSS in MEM (Figure 4.2, panel b). The CSC treated cells by contrast were smaller in size with less cytoplasm; the nucleus was smaller and darker staining, although a percentage of the retracted cells had a nucleus resembling that of the control cells (whole: Figure 4.2, panel c, and vapour: Figure 4. 2, panel d). Epithelial cells treated with condensate are smaller in size and darker staining at light microscopy. Using S.E.M. it is possible to study cell surface changes and shape change in greater detail.

Protocol of Aqueous CSC Exposure for Electron Microscopy:- Both A549 and BEA-2B epithelial cells were trypsinized off culture plates as previously described and resuspended in the appropriate culture mediums at 5×10^5 cells ml^{-1} . Aliquots of cell suspension ($500\mu\text{l}$) were added to 24 well tissue culture plates. Each well contained a sterile glass coverslip. The plates were incubated overnight to allow the cells to adhere and spread. The following morning each well was washed twice with PBS ($500\mu\text{l}$) and then either whole or vapour CSC (10%, $500\mu\text{l}$) or HBSS control (10%, $500\mu\text{l}$) or MEM alone ($500\mu\text{l}$) was added to the wells. The cells were incubated for a further 2 hours at 37°C , 5% CO_2 , after which each well was washed twice with PBS to remove the cell debris and 2.5% electron microscopy grade glutaraldehyde in PBS was added to the wells. These cells were then processed for S.E.M. as described above.

Visualisation by S.E.M.:- Unfortunately, there was a degree of crystalline artifact on these preparations but the general shape of the cells

was clear. Either type of cell treated with MEM alone presented as a well spread and flattened cell (Figure 4.8. panel a is representative of both A549 epithelial cells and BEA-2B epithelial cells). The HBSS had no effect on these cells compared to controls, as seen in Figure 4.8. panel b, which again is representative of the appearance of both A549 epithelial cells and BEA-2B epithelial cells. Upon exposure to either whole or vapour CSC both type cells had rounded up. Figure 4.8. panel c shows the effect of whole CSC and panel d the effect of vapour CSC.

4.3. EFFECTS OF AQUEOUS CSC ON A549 EPITHELIAL CELL SIZE

It is possible to quantify the changes in cell size observed following treatment with CSC using a "spreading assay". To determine if there was a gradual or immediate change in cell size, a series of measurements were made. These experiments were carried out using A549 epithelial cells only.

The spreading assay gives a measurement of size of the epithelial cells which remained attached after exposure to CSC. It must be remembered that this assay is not concerned with changes in cell number or density and cannot take into account the cells that have already detached or those which have been lysed.

Protocol:- A549 epithelial cells were set up as in Section 4.1.1. for visualisation by light microscopy and exposed to whole CSC, vapour CSC or HBSS (10%), with an MEM control also included. In 3 separate experiments, 200 cells were measured for each duplicate sample with each treatment, at each time point of 30 minutes, 1,2 and 4 hours.

As previously mentioned (Section 2.2.3e.), the maximum diameter of

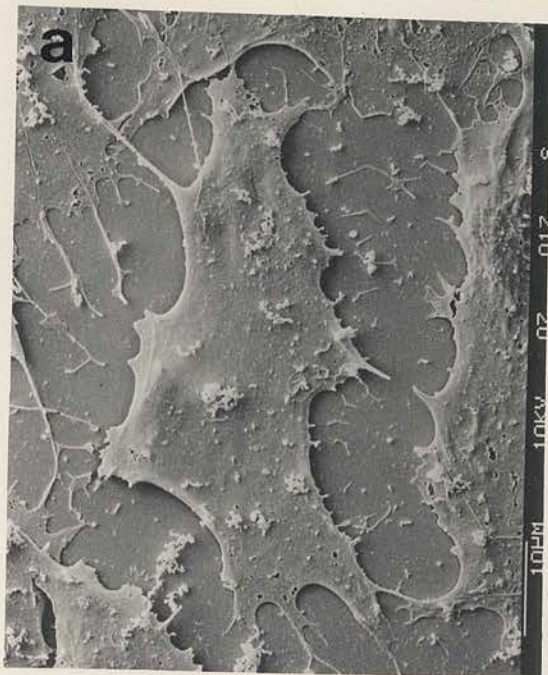


Figure 4.8. S.E.M. image of attached epithelial cells treated with a) MEM, b) 10% HBSS, c) 10% whole CSC, and d) 10% vapour CSC for 2 hours. Note the well spread flattened appearance of the MEM or HBSS treated cells (panel a and b), while the CSC treated cells have rounded up in comparison (panel c and d). (Magnification a) x1.08k; b) x1.3k; c) x1.5k d) x1.3k)

these cells was measured, which was defined as the distance between the two points furthest apart in the cell. These cells were then incubated for up to 4 hours at 37°C, 5% CO₂.

Results:- Figure 4.9. summarises the mean maximum diameters for each treatment at these 4 different time points for the 3 experiments. The mean maximum diameter of A549 epithelial cells in MEM alone stayed constant over the 4 hours of this experiment (Figure 4.9. panel a), and were comparable to values for A549 epithelial cells treated with 10% HBSS in MEM. Thus the aqueous solvent HBSS alone did not effect cell size. When vapour CSC was added there was an obvious and significant decrease in the mean maximum diameter at the second time point of 1 hour (Figure 4.9. panel d). These cells after treatment with either of the CSC became progressively smaller with time and by 4 hours the cells in both cases had a mean maximum diameter which was approximately half the value of the control cells. This mean maximum diameter is a gross measure of cell size. To assess more subtle changes following CSC it is necessary to look at the overall distribution of cell size. Both incubation with MEM or MEM containing 10% HBSS on cells over time gave similar results (Figure 4.10. panel a & b). The distribution of cell size in both cases was approximately normal bell shaped for each time point but was slightly skewed towards higher values. The range of maximum diameters in these treatments was from 10µm to 138µm with the majority of cells having a maximum diameter of between 30µm and 85µm. Compared with control cells there was a marked shift in the frequency of distributions of whole and vapour CSC treated cells (Figure 4.11, panel a & b). This shift in the frequency distribution was time dependent. At the 30 minute and 1 hour time points both for whole and vapour CSC

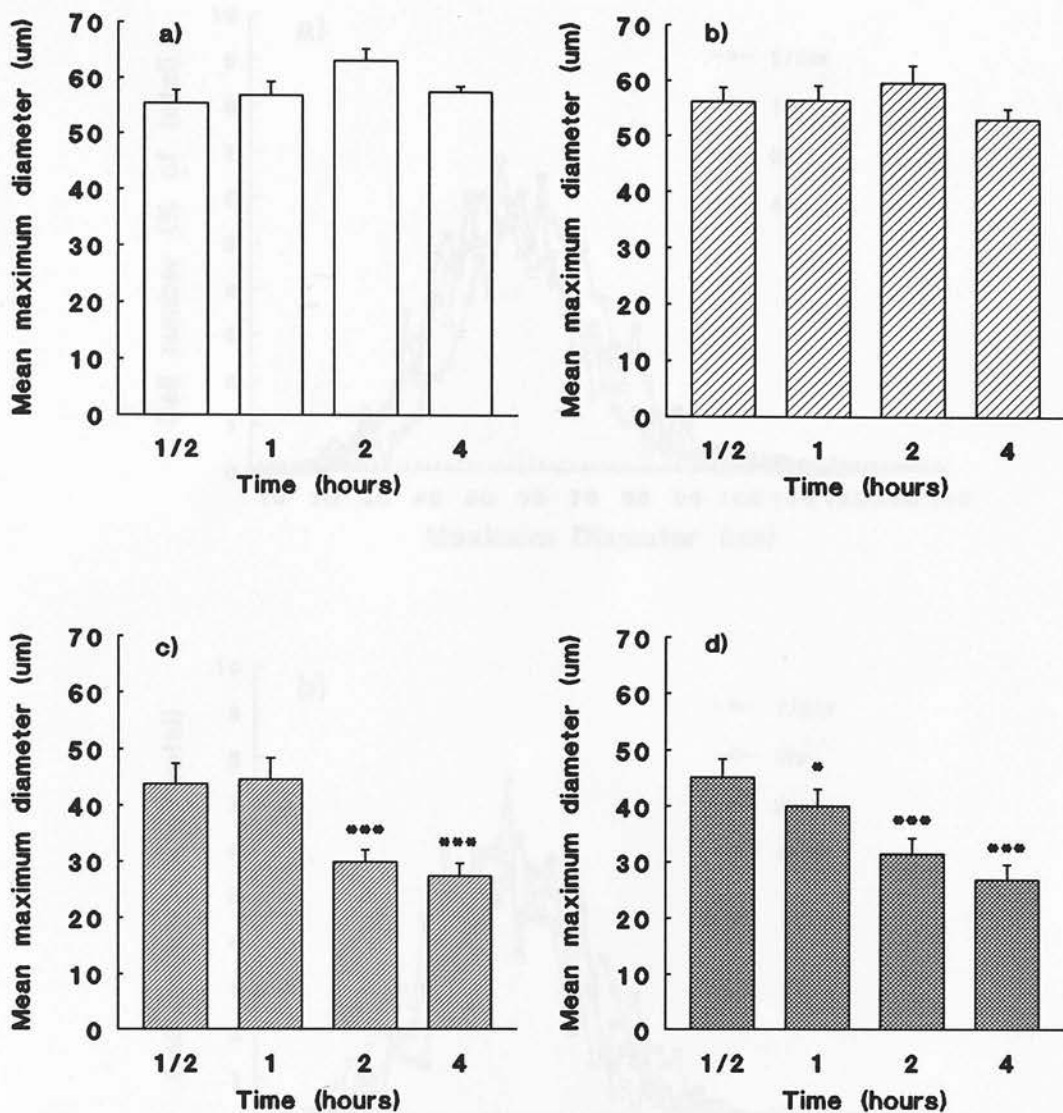


Figure 4.9. The effect of a) MEM, b) 10% HBSS, c) 10% whole CSC, and d) 10% vapour on the mean maximum diameter of attached A549 epithelial cells over 4 hours, measured in μm . The histograms represent the mean of three separate experiments, each with duplicate samples of 200 cells, and the bars indicate 1 SEM in each case. * $p < 0.05$; *** $p < 0.001$ compared with either MEM or HBSS at that time point.

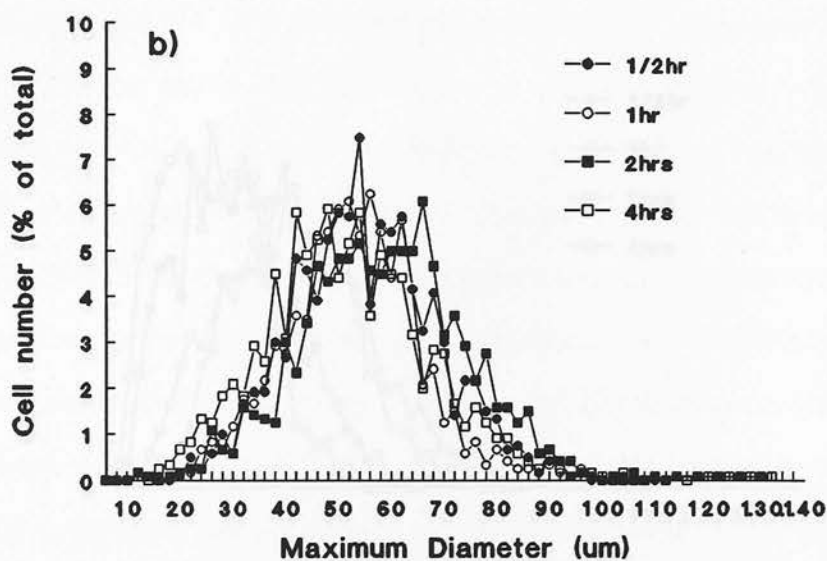
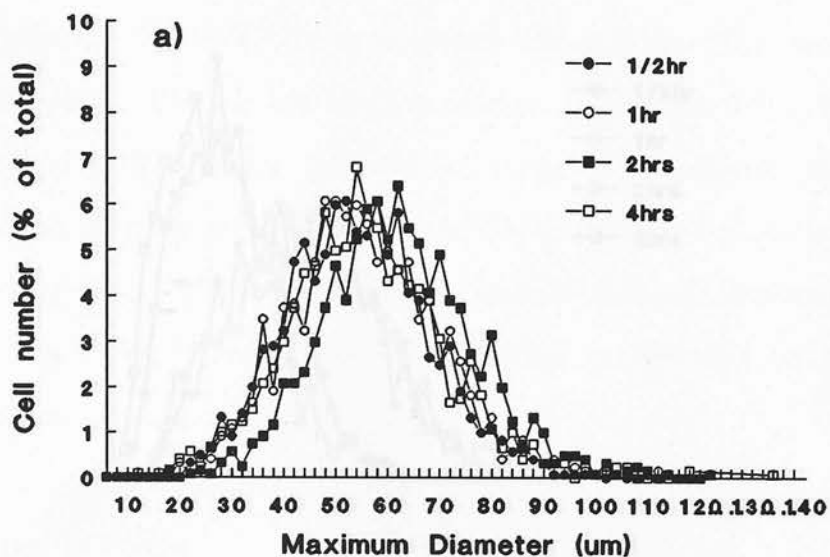


Figure 4.10. The effect of a) MEM, and b) 10% HBSS on the distribution of the mean maximum diameter of attached A549 epithelial cells over time; 30 minutes (●), 1 hour (○), 2 hours (■), and 4 hours (□). The data is the accumulated results of duplicate samples of 200 cells per time point in three separate experiments.

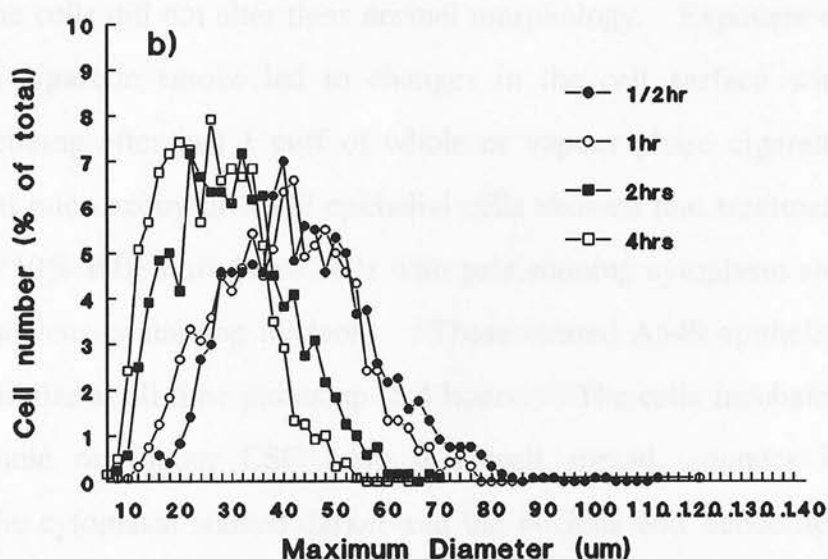
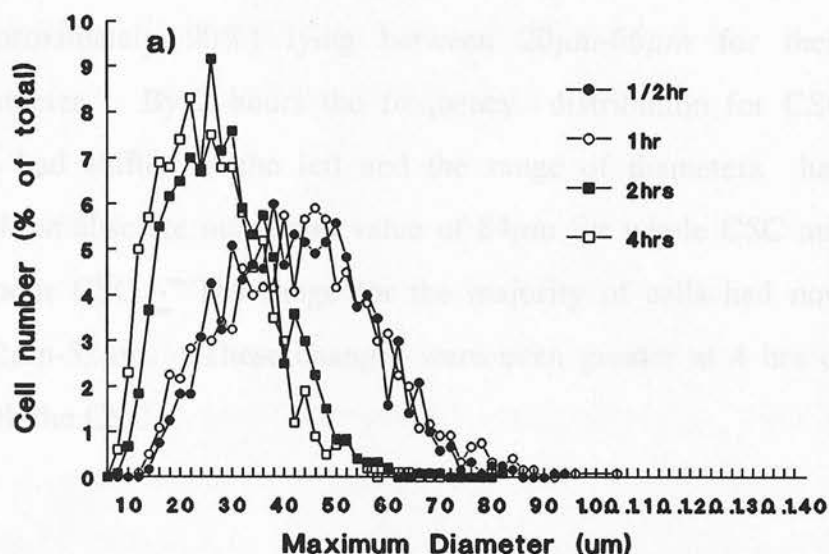


Figure 4.11. The effect of a) 10% whole CSC, and b) 10% vapour CSC on the distribution of the mean maximum diameter of attached A549 epithelial cells over time; 30 minutes (●), 1 hour (○), 2 hours (■), and 4 hours (□). The data is the accumulated results of duplicate samples of 200 cells per time point in three separate experiments.

treated cells, a normal distribution was still present, although the range of maximum diameters was now between $6\mu\text{m}$ and $118\mu\text{m}$ with the majority of cells (approximately 90%) lying between $20\mu\text{m}$ - $66\mu\text{m}$ for their maximum diameter. By 2 hours the frequency distribution for CSC exposed cells had shifted to the left and the range of diameters had decreased with an absolute maximum value of $84\mu\text{m}$ for whole CSC and $70\mu\text{m}$ for vapour CSC. The range for the majority of cells had now dropped to $12\mu\text{m}$ - $52\mu\text{m}$. These changes were even greater at 4 hrs of incubation with the CSC.

SUMMARY

The A549 epithelial cell in suspension had a ruffled membrane, sham exposure of the cells did not alter their normal morphology. Exposure of these cells to cigarette smoke led to changes in the cell surface with membrane blebbing after just 1 puff of whole or vapour phase cigarette smoke. Light microscopy of A549 epithelial cells showed that treatment with MEM or 10% HBSS produced cells with pale staining cytoplasm and a prominent nucleus containing nucleoli. These treated A549 epithelial cells looked similar at all time points up to 4 hours. The cells incubated with both whole or vapour CSC were less well spread, rounder in appearance, the cytoplasm stained darker and the nucleus was condensed in the majority of the cells. Similar changes in the light microscopic appearance of BEA-2B cells treated with CSC were observed. Examination by S.E.M. of CSC treated A549 or BEA-2B cells showed a much rounder cell compared with the flattened well spread untreated cells.

CHAPTER 5

PROTECTIVE POTENTIAL OF ANTIOXIDANTS AND ANTIPROTEINASES

The epithelial cells lining the airspaces of the lung exist in a potentially toxic environment. This is particularly true of smokers in whom oxidants may be released from airspace leukocytes or inhaled in cigarette smoke. Airspace leukocytes may also be activated to release proteases. To cope with this the lung has developed a protective system of antioxidants and antiproteinases. In order to assess the mechanism of smoke-induced epithelial injury the protective capacity of specific antioxidants was investigated in relation to the epithelial cell injury caused by cigarette smoke and its CSC's, which has been already described in Chapter 3, and compared to the protection conveyed by an proteinase inhibitor. In the functional assays described previously of detachment and proliferation, the specific enzymatic antioxidants of SOD and catalase and the general non-enzymatic antioxidant GSH were compared with the antiproteinase inhibitor α -1PI. The intra and extracellular antioxidant potential of GSH was studied in depth in the detachment assay as it was possible experimentally to deplete or enhance the GSH concentration in cells in this system. The effect of this general antioxidant was also examined in the attachment assay system.

Inhibition of the Glutathione Redox System

Depletion of intracellular reduced glutathione can be produced by reaction with electrophilic agents diethylmaleate (DEM) (Chasseaud, 1979; Wedner *et al*, 1981) or 1-chloro-2,4-dinitrobenzene (DNB)

(Novogrodsky *et al*, 1979; Arrick *et al*, 1982) to form thioether conjugates. Glutathione reductase may be selectively inhibited by 1,3-bis (chloroethyl)-1-nitrosourea (BCNU) (Frischer & Ahmad, 1977; Babson & Reed, 1978). L-Buthionine-SR-Sulfoximine (BSO) is an agent which inhibits -glutamyl cysteine synthetase without producing any in vivo toxic effect (Griffith, 1982); -glutamylcysteine synthetase catalyzes the phosphorylation of BSO by MgATP, and so buthionine sulfoximine phosphate is tightly bound to the fully inhibited enzyme (Griffith, 1982). BSO has been shown to block glutathione synthesis in vitro in A549 alveolar epithelial cells (Brodie & Reed, 1985). The glutathione content of the cell line was reduced to 20% of control values by incubation with 10 μ M BSO for 24 hours by Kang & Enger (1990).

Augmentation of the Glutathione Redox System

The intracellular levels of GSH can be enhanced by the addition of the amino acid precursor L-cysteine or its derivative N-Acetylcysteine (Issels *et al*, 1988; Phelps *et al*, 1992).

Cysteine is a precursor in the biosynthesis of the antioxidant glutathione (Meister & Anderson, 1983; Bergstrand *et al*, 1986) which can be produced in the liver. NAC has been shown to raise intracellular GSH stores (Berggren *et al*, 1984; De Flora *et al*, 1985).

5.1. EPITHELIAL ATTACHMENT

Only A549 epithelial cells were used in the following experiments.

5.1.1. Effect of GSH on Cigarette Smoke Exposed A549 Epithelial Cells

The protective effects of the antioxidant GSH against cigarette smoke exposure of cells was examined in the attachment assay. Only vapour

phase cigarette smoke was used for the reasons given in Section 3.

Protocol:- The A549 epithelial cells were trypsinized off the culture flasks and ^{51}Cr labelled as described in Section 2.2.1. The cells were resuspended at 1×10^6 cells ml^{-1} in MEM with or without a range of GSH (50-400 μM). MEM cell suspensions were subsequently exposed to 4 puffs of vapour phase cigarette smoke (Section 2.4.), washed immediately in MEM (200g, 5 minutes), and the effects on cell attachment were measured (Section 2.1.).

Results:- Glutathione alone, at concentrations up to 400 μM , did not affect cell attachment (Figure 5.1, panel a). The reduction in cell attachment following exposure to cigarette smoke was not significantly altered by the addition of GSH in concentrations up to 100 μM (Figure 5.1, panel b). However, cells suspended in medium to which GSH (150-400 μM) had been added did not have their attachment compromised by exposure to vapour phase cigarette smoke.

5.2. EPITHELIAL DETACHMENT

The protective potential of the antioxidants and the antiprotease against CSC induced A549 epithelial cell damage were also investigated in the epithelial detachment assay. The specific inhibitors SOD, catalase, desferrioxamine and the protease inhibitors α -1PI and soya bean trypsin inhibitor (SBTI) were also examined for their ability to protect against the effect of cigarette smoke when present extracellularly. The general antioxidant GSH was assessed for its role both intra- and extracellularly.

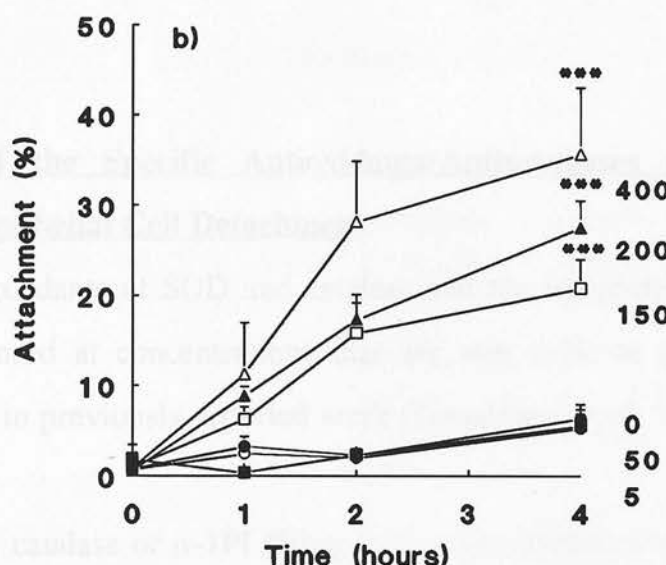
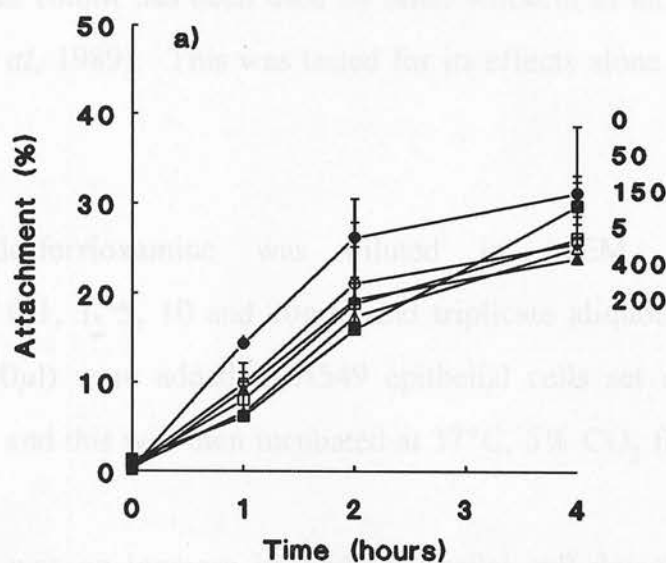


Figure 5.1. The effect of increasing concentrations of GSH; control (●), 5uM (○), 50uM (■), 150uM (□), 200uM (▲), and 400uM (△) on the attachment of a) sham, and b) vapour phase cigarette smoke exposed A549 epithelial cells over 4 hours, measured in percentage of ^{51}Cr that is cell associated. The data is the mean of triplicate wells in five separate experiments and the bars indicate 1 SEM in each case. *** $p < 0.001$ compared with control smoke exposed.

5.2.1. Effect of Desferrioxamine on A549 Epithelial Cell Detachment

Desferrioxamine at 10mM has been used by other workers as an inhibitor of $\cdot\text{OH}$ (Churg *et al*, 1989). This was tested for its effects alone on A549 epithelial cells.

Protocol:- The desferrioxamine was diluted in MEM to give concentrations of 0.1, 1, 5, 10 and 20mM and triplicate aliquots of each concentration (200 μl) were added to A549 epithelial cells set up in the detachment assay and this was then incubated at 37°C, 5% CO_2 for 4 hrs.

Results:- There was no increase in A549 epithelial cell detachment or cell lysis over control values of the cell treated with desferrioxamine (Figure 5.2).

5.2.2. Effect of the Specific Antioxidants/Antiproteases on CSC Induced A549 Epithelial Cell Detachment

The specific antioxidants of SOD and catalase and the antiproteinase, α -1PI, have been used at concentrations that are non toxic to the A549 epithelial cells as in previously reported work (Donaldson *et al*, 1993).

Protocol:- SOD, catalase or α -1PI (50 $\mu\text{g}.\text{ml}^{-1}$) were combined with each type of condensate (organic CSC, whole CSC or vapour CSC) and incubated in the A549 epithelial detachment assay at 37°C, 5% CO_2 for 4 hours. In addition desferrioxamine (10 μM) or SBTI (1mg. ml^{-1}) were combined with either whole CSC or vapour CSC and examined for their protective potential in the A549 epithelial detachment assay. The organic CSC was used at a 2% dilution while a 10% dilution of aqueous CSC was the standard used for the following experiments.

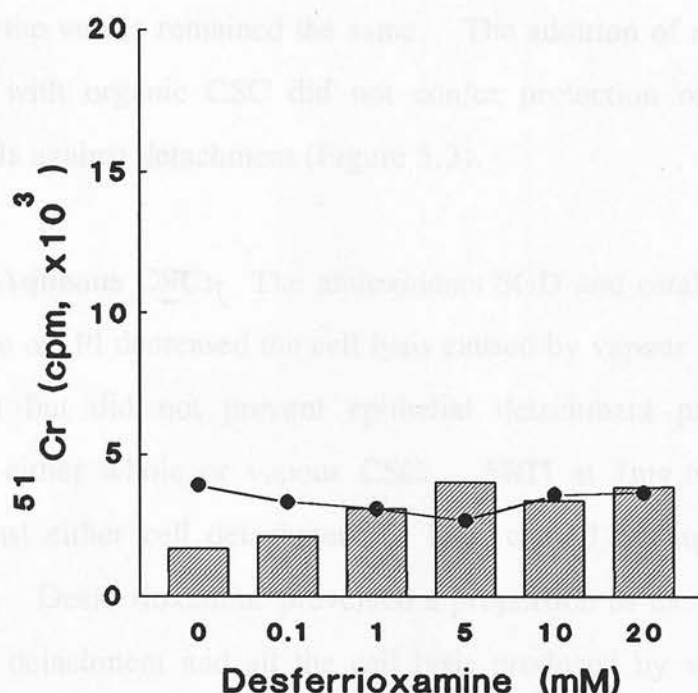


Figure 5.2. The effect of increasing concentrations of desferrioxamine on the detachment (histograms) and lysis (●) of A549 epithelial cells.

Results for Organic CSC:- SOD, catalase or α -1PI alone did not cause any changes in detachment or lysis above background and when combined with DMSO the values remained the same. The addition of any of these components with organic CSC did not confer protection of the A549 epithelial cells against detachment (Figure 5.3).

Results for Aqueous CSC:- The antioxidants SOD and catalase and the antiproteinase α -1PI decreased the cell lysis caused by vapour CSC (10%) (Figure 5.4) but did not prevent epithelial detachment produced by exposure to either whole or vapour CSC. SBTI at 1mg.ml^{-1} did not protect against either cell detachment or lysis caused by aqueous CSC (Figure 5.5). Desferrioxamine prevented a proportion of the whole CSC induced cell detachment and all the cell lysis produced by vapour CSC (Figure 5.6).

5.2.3. Effect of GSH, Cysteine and NAC on A549 Epithelial Cell Detachment

GSH, Cysteine and NAC were all used in the detachment assay as potential protectors against cell injury. A range of concentrations of each of these compounds was tested in the detachment assay for potential harmful effects. These results then gave a concentration at which the appropriate compound could be used in the detachment assay without itself being toxic.

Protocol:- GSH at 200, 400, 800, and $1600\mu\text{M}$ ($200\mu\text{l}$ aliquots), cysteine at 0.1, 0.5, 1 and 10mM ($200\mu\text{l}$ aliquots) and NAC 0.1, 0.5, 1 and 10mM ($200\mu\text{l}$ aliquots) were added to A549 epithelial cells in the detachment assay (Section 2.2.3b.) and incubated for 4 hours.

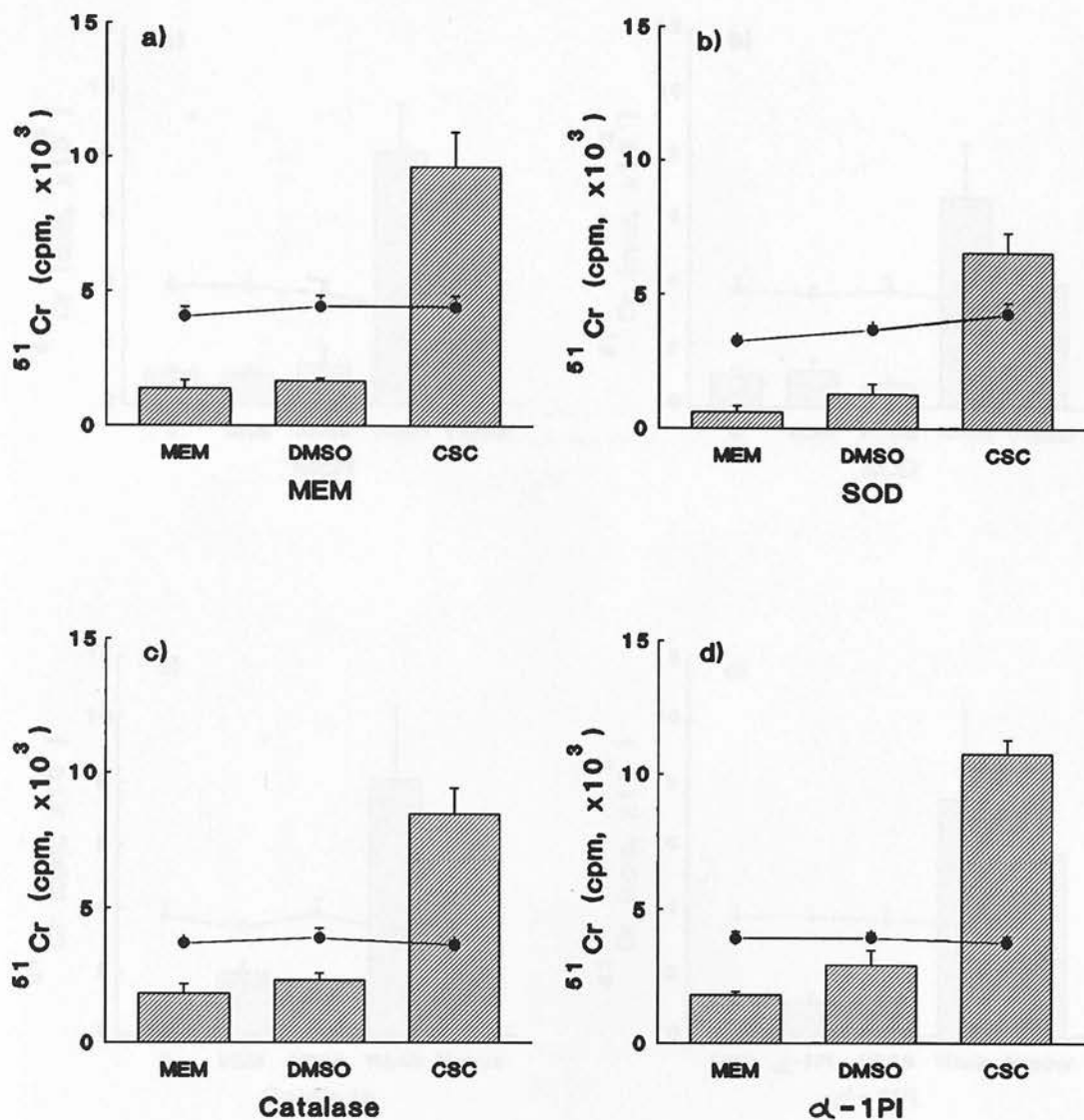


Figure 5.3. The effect of addition of a) MEM, b) SOD, c) catalase, and d) α -1PI on the detachment (histograms) and lysis (\bullet) of A549 epithelial cells induced by 2% DMSO or organic CSC, measured in ^{51}Cr cpm. The histograms/ \bullet represent the mean of triplicate wells in three separate experiments and the bars indicate 1 SEM in each case. There was no significant change in detachment or lysis with the addition of SOD, catalase or α -1PI.

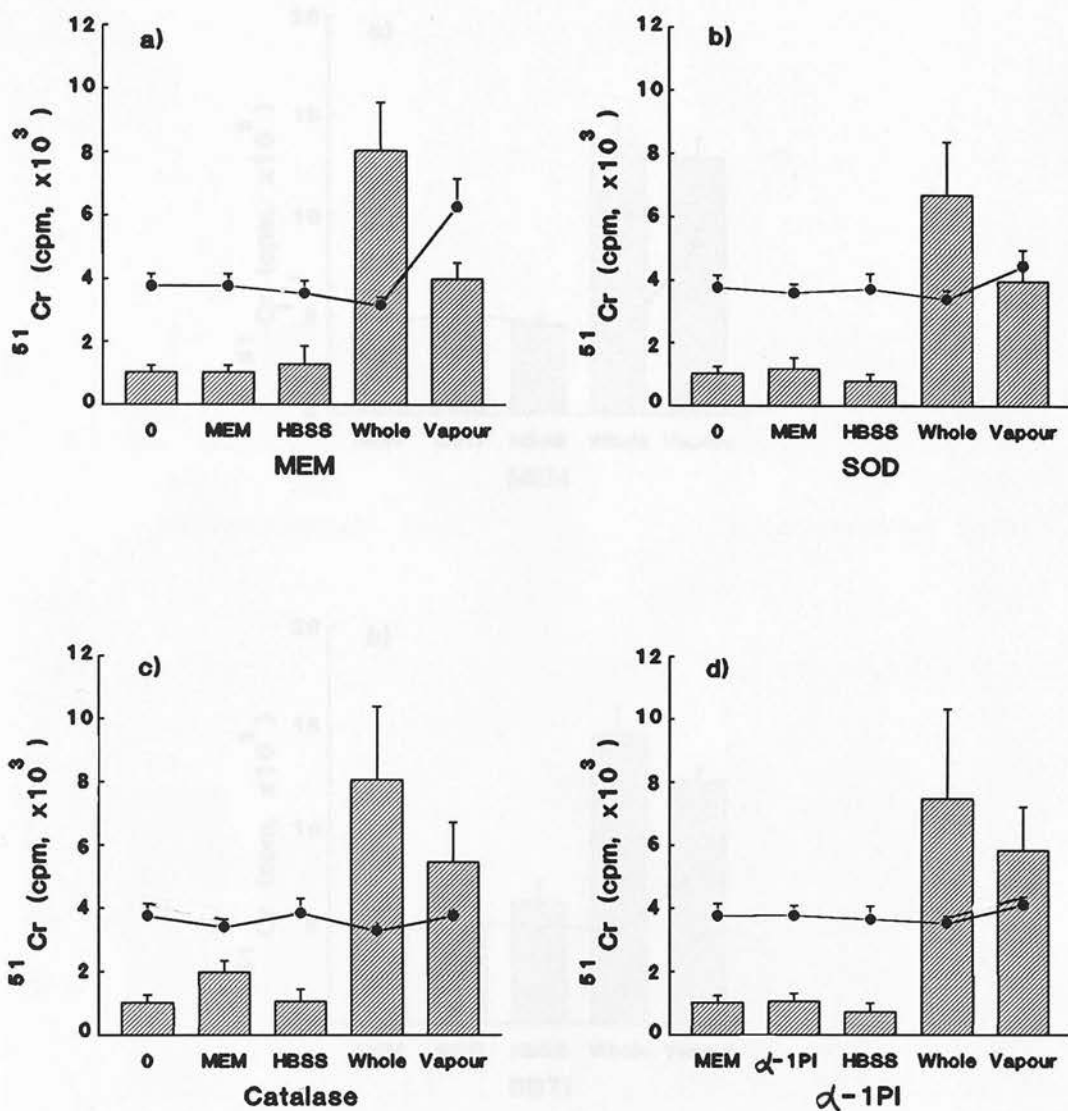


Figure 5.4. The effect of addition of a) MEM, b) SOD, c) catalase, and d) α -1PI on the detachment (histograms) and lysis (\bullet) of A549 epithelial cells induced by 10% HBSS, whole or vapour phase CSC, measured in ^{51}Cr cpm. The histograms/ \bullet represent the mean of triplicate wells in three separate experiments and the bars indicate 1 SEM in each case.

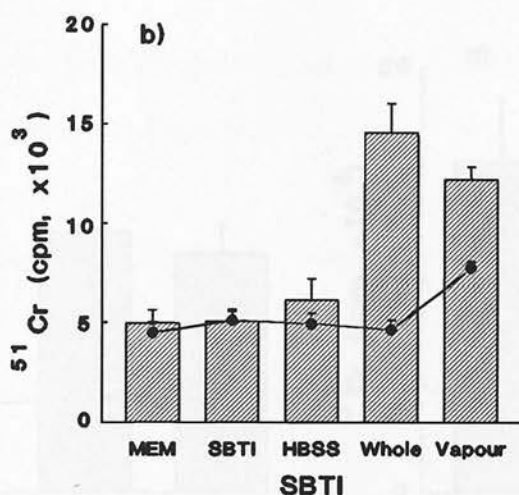
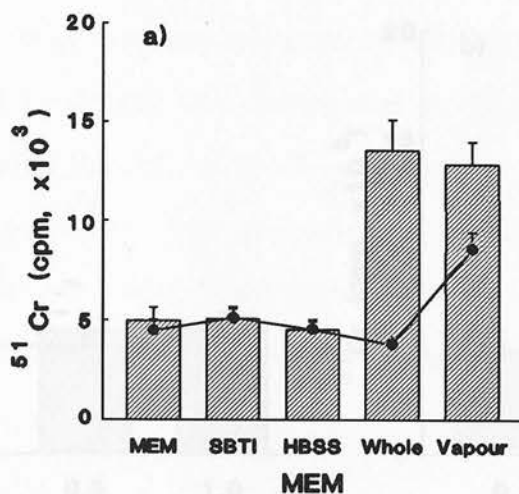


Figure 5.5. The effect of addition of a) MEM, and b) SBTI on the detachment (histograms) and lysis (—●—) of A549 epithelial cells induced by 10% HBSS, whole or vapour CSC, measured in ⁵¹Cr cpm. The histograms/—●— represent the mean of triplicate wells in four separate experiments and the bars indicate 1 SEM in each case. There was no significant change in detachment or lysis with the addition of SBTI.

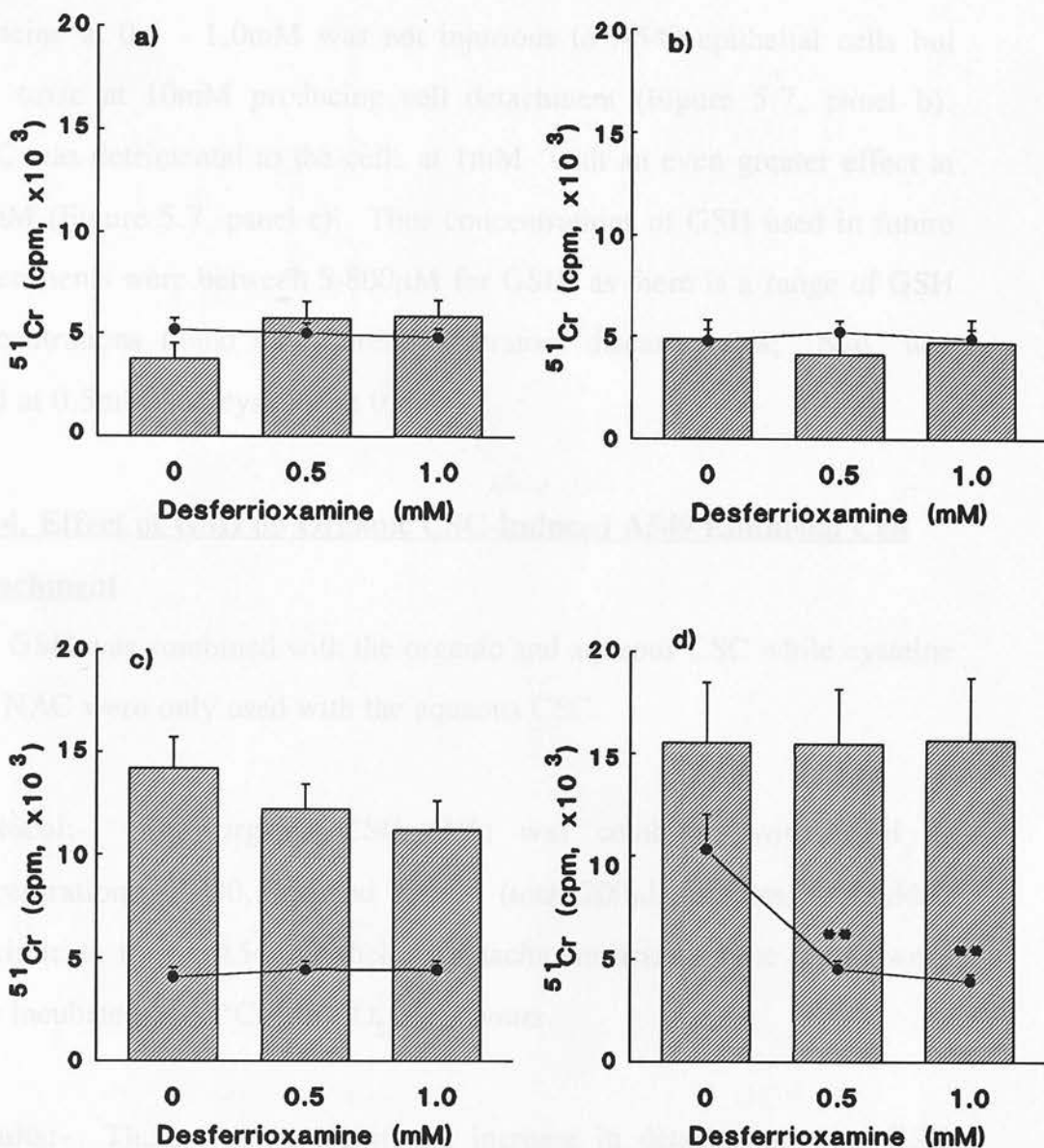


Figure 5.6. The effect of desferrioxamine on a) MEM, b) 10% HBSS, c) 10% whole CSC, and d) 10% vapour CSC induced detachment (histograms) and lysis (\bullet) of A549 epithelial cells measured in ^{51}Cr cpm. The histograms/ \bullet represent the mean of six separate experiments and the bars indicate 1 SEM in each case. * $p < 0.05$; ** $p < 0.01$ compared with CSC alone (0).

Results:- GSH was found to be non cytotoxic up to 1600 μ M (Figure 5.7, panel a) and did not cause any excess detachment at these concentrations. Cysteine at 0.1 - 1.0mM was not injurious to A549 epithelial cells but was toxic at 10mM producing cell detachment (Figure 5.7, panel b). NAC was detrimental to the cells at 1mM with an even greater effect at 10mM (Figure 5.7, panel c). Thus concentrations of GSH used in future experiments were between 5-800 μ M for GSH, as there is a range of GSH concentrations found in different respiratory disease states; NAC was used at 0.5mM and cysteine at 0.5mM.

5.2.4. Effect of GSH on Organic CSC-Induced A549 Epithelial Cell

Detachment

The GSH was combined with the organic and aqueous CSC while cysteine and NAC were only used with the aqueous CSC.

Protocol:- The organic CSC (2%) was combined with GSH at concentrations of 200, 400 and 800 μ M (total 200 μ l, aliquots) and added in triplicate to the A549 epithelial detachment assay; these plates were then incubated at 37°C, 5% CO₂ for 4 hours.

Results:- There was no significant increase in detachment with GSH alone or in combination with the carrier DMSO. GSH in any concentration failed to protect against the A549 epithelial cell detachment caused by the organic CSC (Figure 5.8).

5.2.5. Effects of GSH, Cysteine or NAC on Aqueous CSC Induced A549 Epithelial Cell Detachment

Protocol:- The aqueous CSC's (10%) were combined with the full range

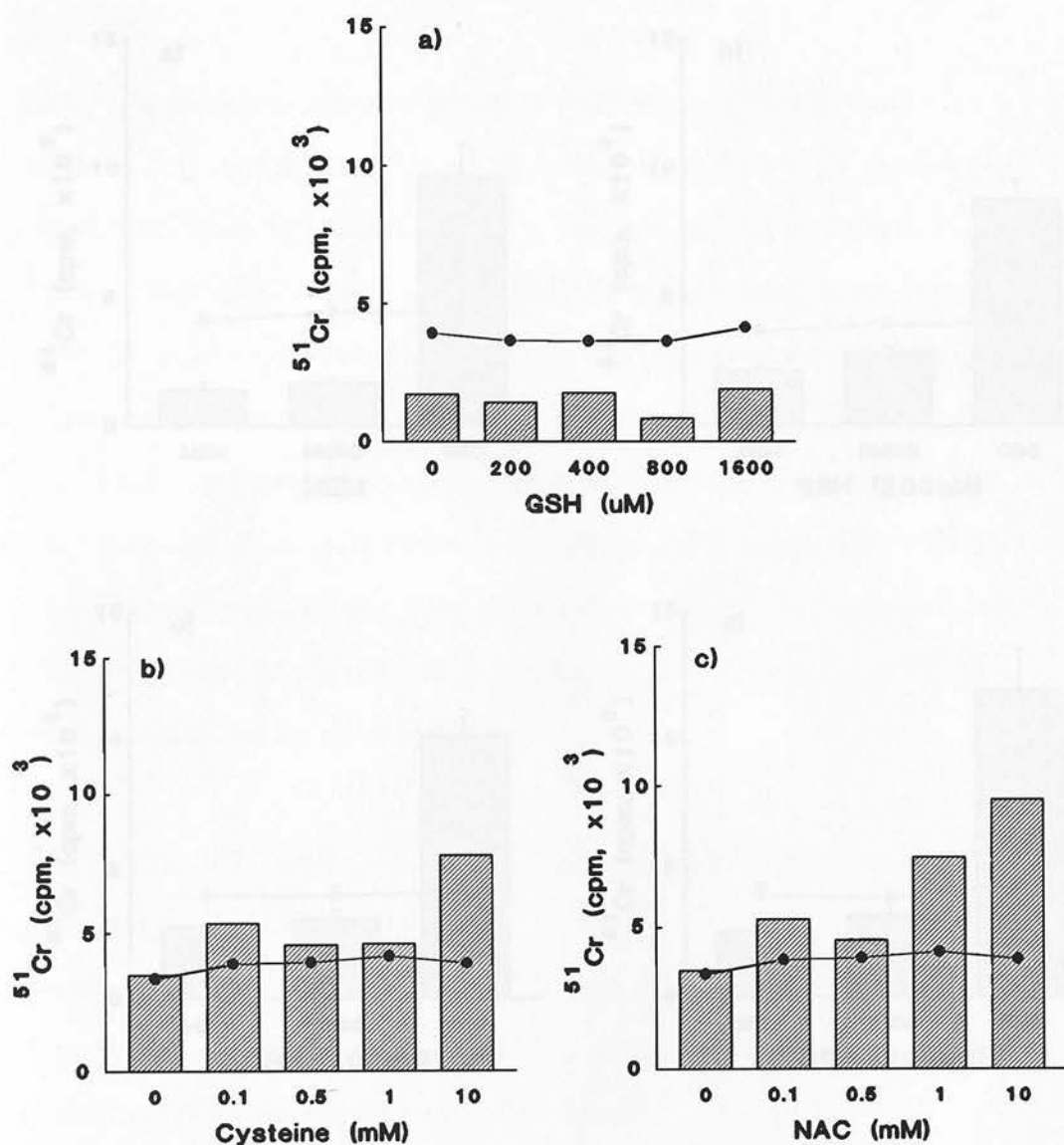


Figure 5.7. The effect of increasing concentrations of a) GSH, b) Cysteine, and c) NAC on the detachment (histograms) and lysis (●) of A549 epithelial cells, measured in ^{51}Cr cpm. The histograms are the result of triplicate wells in one experiment.

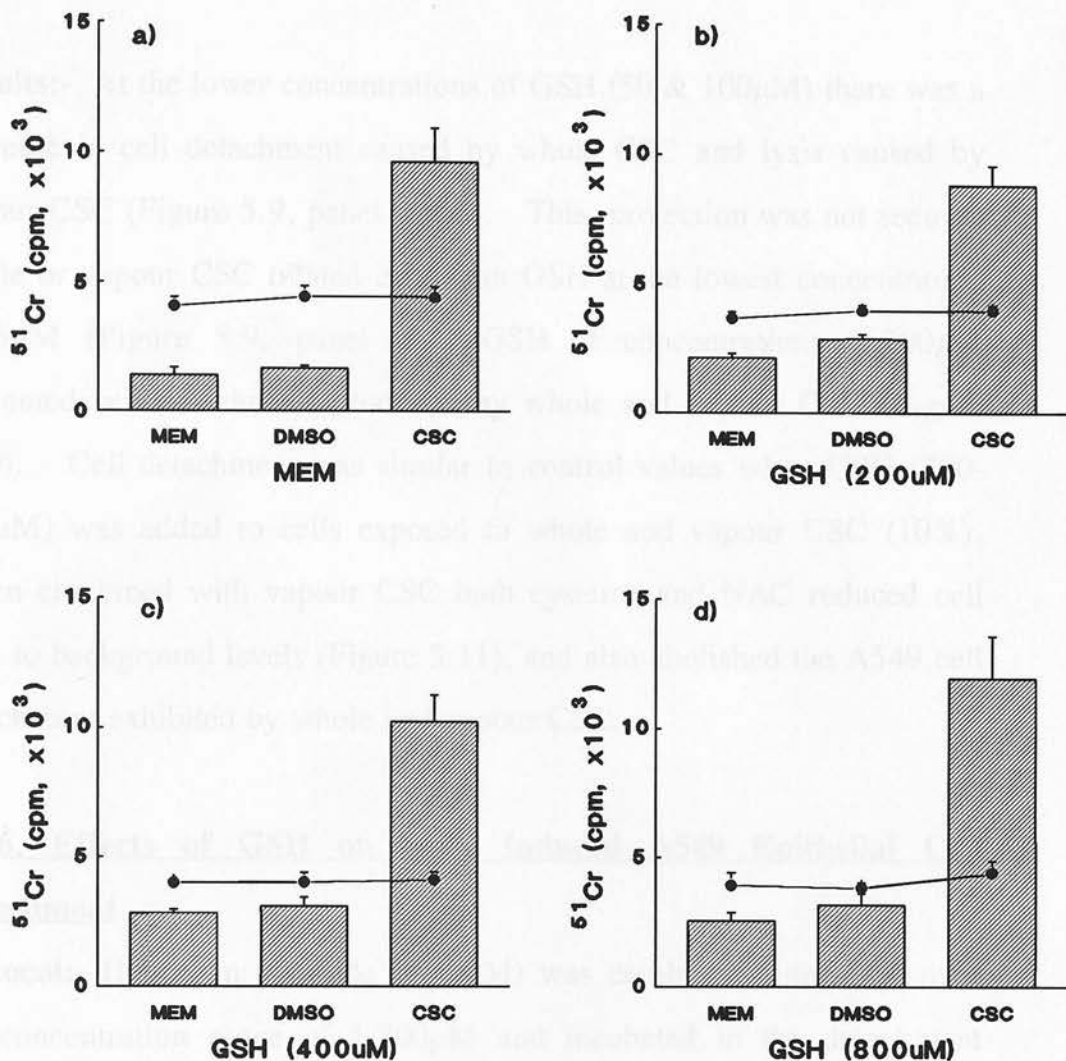


Figure 5.8. The effect of the addition of a) MEM, b) 200uM GSH, c) 400uM GSH, and d) 800uM GSH on the detachment (histograms) and lysis (\bullet) of A549 epithelial cells induced by 2% DMSO or organic CSC, measured in ^{51}Cr cpm. The histograms/ \bullet represent the mean of triplicate wells in three separate experiments and the bars indicate 1 SEM in each case. There was no significant change in detachment or lysis with the addition of any of the concentrations of GSH.

of GSH concentrations (5-800 μ M) in the A549 epithelial detachment assay (total 200 μ l aliquots), and incubated at 37°C, 5% CO₂ for 4 hours.

Results:- At the lower concentrations of GSH (50 & 100 μ M) there was a decrease in cell detachment caused by whole CSC and lysis caused by vapour CSC (Figure 5.9, panel c & d). This protection was not seen on whole or vapour CSC treated cells with GSH at the lowest concentration of 5 μ M (Figure 5.9, panel b). GSH at concentrations \geq 200 μ M prevented cell detachment produced by whole and vapour CSC (Figure 5.10). Cell detachment was similar to control values when GSH (200-800 μ M) was added to cells exposed to whole and vapour CSC (10%). When combined with vapour CSC both cysteine and NAC reduced cell lysis to background levels (Figure 5.11), and also abolished the A549 cell detachment exhibited by whole and vapour CSC.

5.2.6. Effects of GSH on H₂O₂ Induced A549 Epithelial Cell Detachment

Protocol:- Hydrogen peroxide (5-50 μ M) was combined with GSH over the concentration range of 5-800 μ M and incubated in the detachment assay with A549 epithelial cells for 4 hours at 37°C, 5% CO₂.

Results:- H₂O₂ produced a dose dependent increase in A549 epithelial cell detachment and cell lysis (Section 3.3.4.). This detachment and lysis could be partially or totally protected against, depending upon the concentration of GSH in co-incubation. At the top dose of 50 μ M H₂O₂ the GSH, even at the highest doses, could only protect against the cytolytic effects (Figure 5.12). This decrease in cell lysis was dose-dependent, with total protection only occurring with cells treated with 400

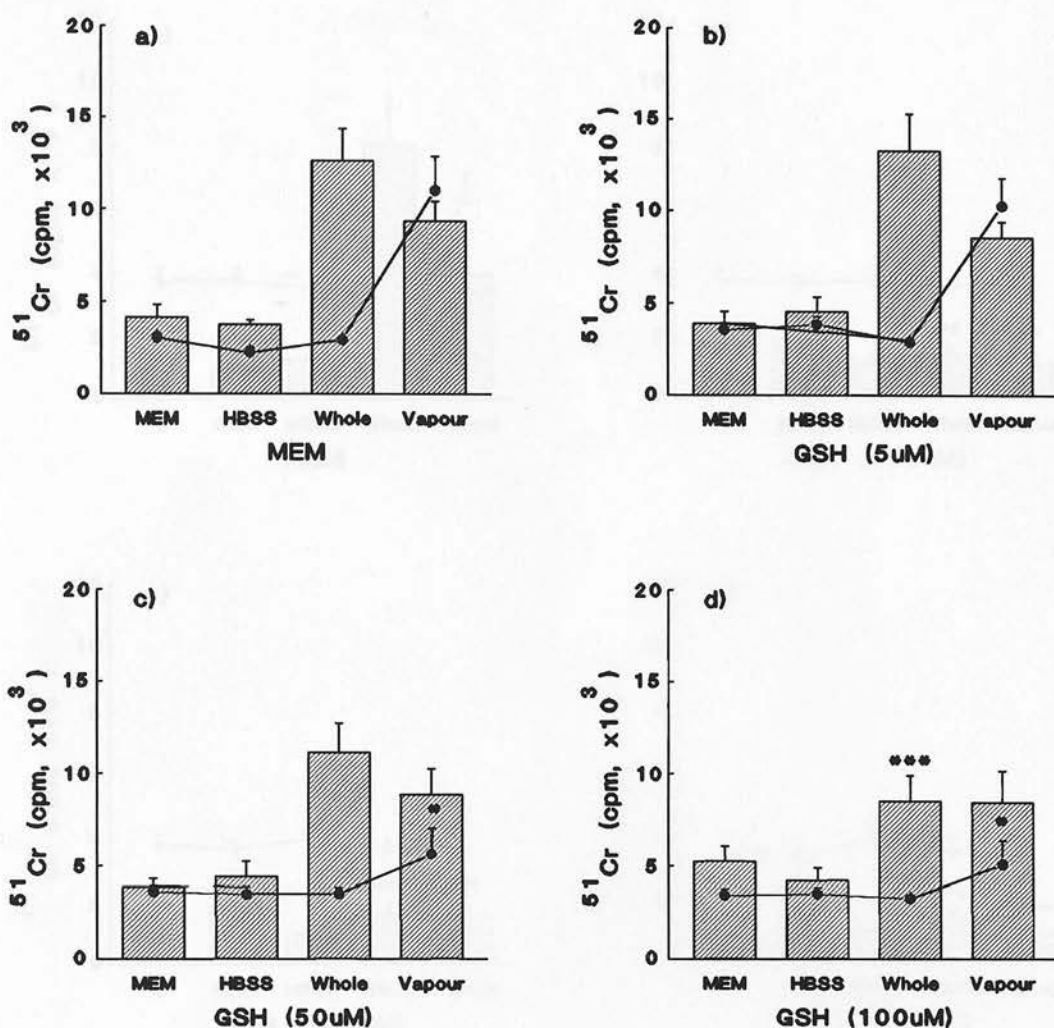


Figure 5.9. The effect of addition of a) MEM, b) 5uM GSH, c) 50uM GSH, and d) 100uM GSH on the detachment (histograms) and lysis (—●—) of A549 epithelial cells induced by 10% HBBS, whole or vapour CSC, measured in ^{51}Cr cpm. The histograms/—●— represent the mean of triplicate wells in four separate experiments and the bars indicate 1 SEM in each case. There was significant reduction in cell injury with addition of GSH. * $p < 0.05$; *** $p < 0.001$ compared with CSC alone.

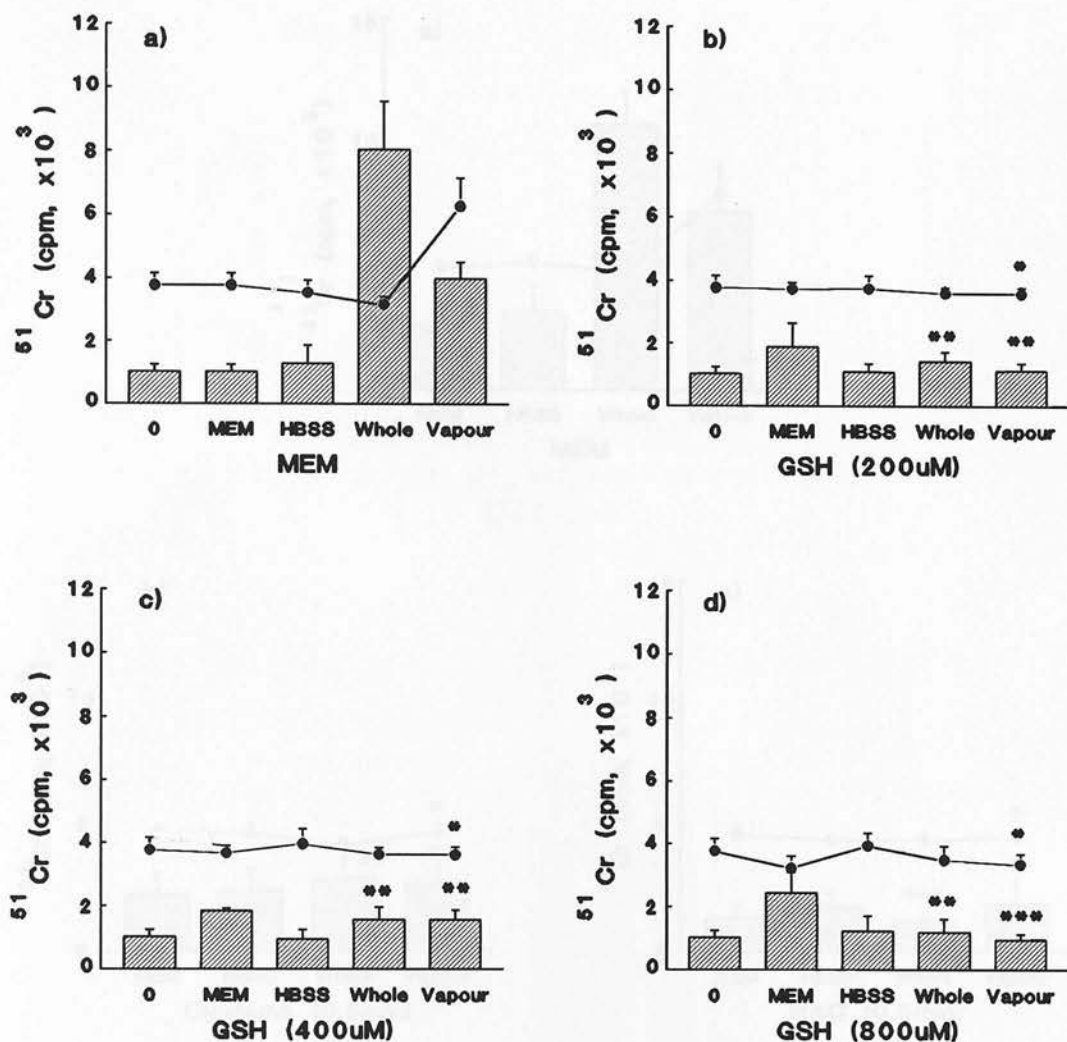


Figure 5.10. The effect of addition of a) MEM, b) 200uM GSH, c) 400uM GSH, and d) 800uM GSH on the detachment (histograms) and lysis (\bullet) of A549 epithelial cells induced by 10% HBSS, whole or vapour CSC, measured in ^{51}Cr cpm. The histograms/ \bullet represent the mean of triplicate wells in four separate experiments and the bars indicate 1 SEM in each case. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared with the CSC alone.

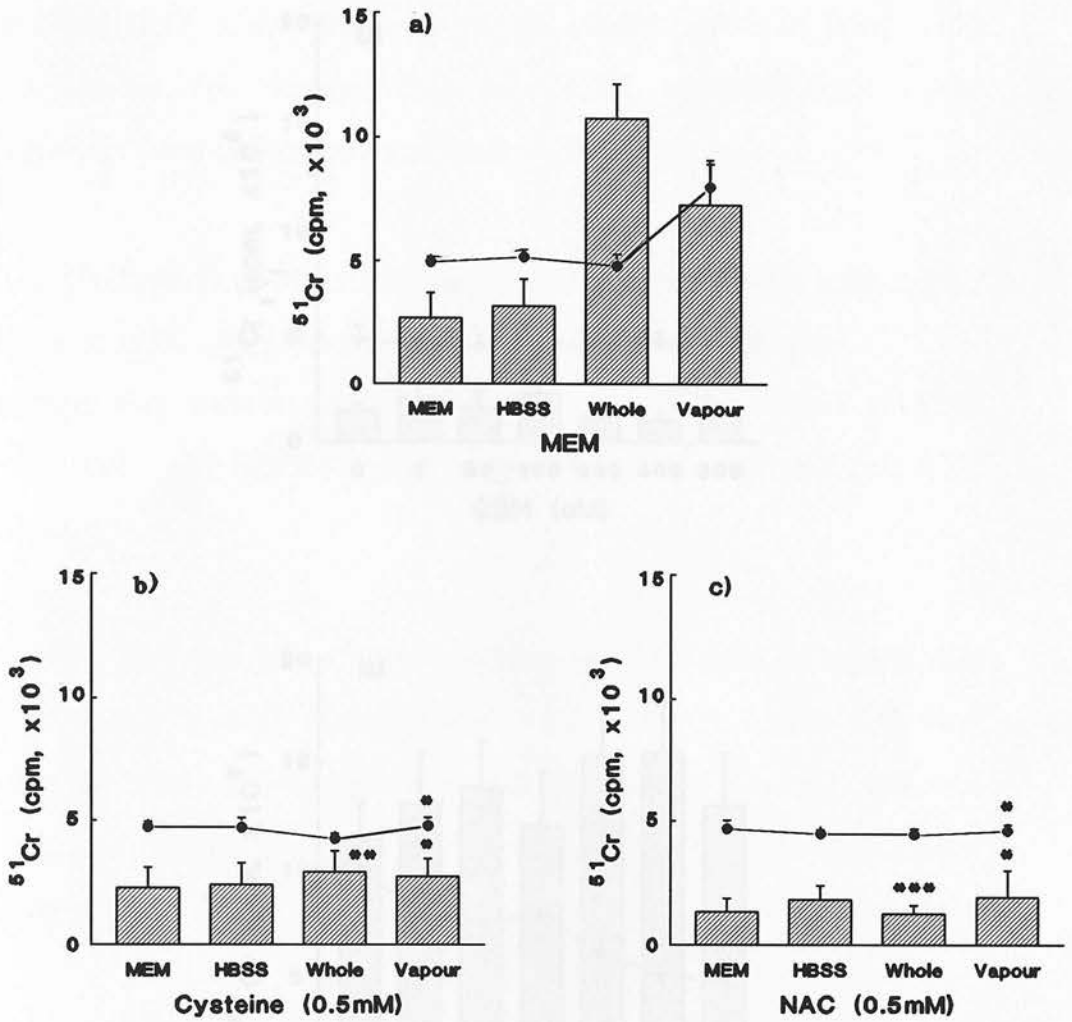


Figure 5.11. The effect of addition of a) MEM, b) 0.5mM Cysteine, and c) 0.5mM NAC on the detachment (histograms) and lysis (●) of A549 epithelial cells induced by 10% HBSS, whole or vapour CSC, measured in ^{51}Cr cpm. The histograms/● represent the mean of triplicate wells in four separate experiments and the bars indicate 1 SEM in each case. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared with CSC alone.

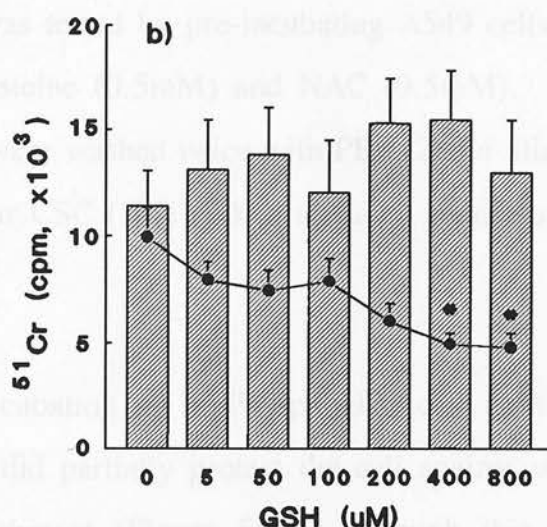
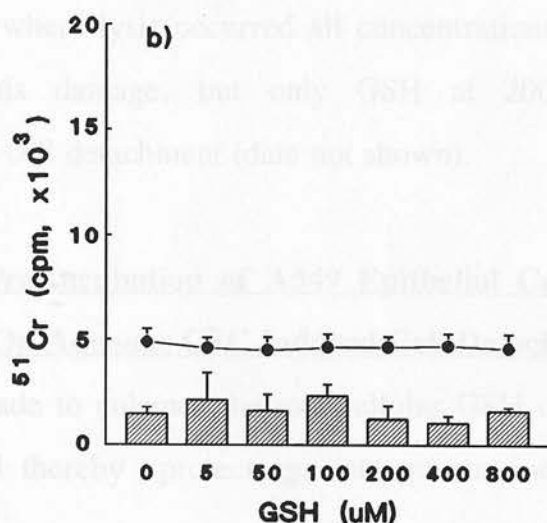


Figure 5.12. The effect of increasing concentrations of GSH on the detachment (histograms) and lysis (\bullet) of A549 epithelial cells induced by a) MEM, and b) 50uM H2O2, measured in ^{51}Cr cpm. The histograms/ \bullet represent the mean of triplicate wells in five separate experiments and the bars indicate 1 SEM in each case. * $p < 0.05$ compared with H2O2 alone (0).

and 800 μ M. Along with this decrease in lysis there was a concurrent rise in the lesser damage of cell detachment. At the lower concentrations of 15-20 μ M H₂O₂ where lysis occurred all concentrations of GSH could protect against this damage, but only GSH at 200-800 μ M could completely prevent cell detachment (data not shown).

5.2.7. Effect of Pre-Incubation of A549 Epithelial Cells with GSH, Cysteine or NAC On Aqueous CSC Induced Cell Detachment

An attempt was made to enhance the intracellular GSH content of A549 epithelial cells and thereby protect against aqueous induced cell CSC detachment.

Protocol:- This was tested by pre-incubating A549 cells overnight with GSH (400 μ M), cysteine (0.5mM) and NAC (0.5mM). The following morning the cells were washed twice with PBS (200 μ l aliquots) and then the whole or vapour CSC (10%, 200 μ l aliquots) added to the detachment assay for 4 hours.

Results:- Pre-incubation of A549 epithelial cell cultures with either cysteine or NAC did partially protect the cell against whole or vapour CSC-induced detachment (Figure 5.13), although this result was not significant.

The A549 cells were initially incubated with GSH in concentrations at 400 μ M which had given protection when present in the extracellular milieu. However, there was no change in vapour CSC induced cell lysis of GSH pretreated cells compared with cells incubated overnight in MEM alone ($p < 0.05$). Furthermore there was also no significant differences

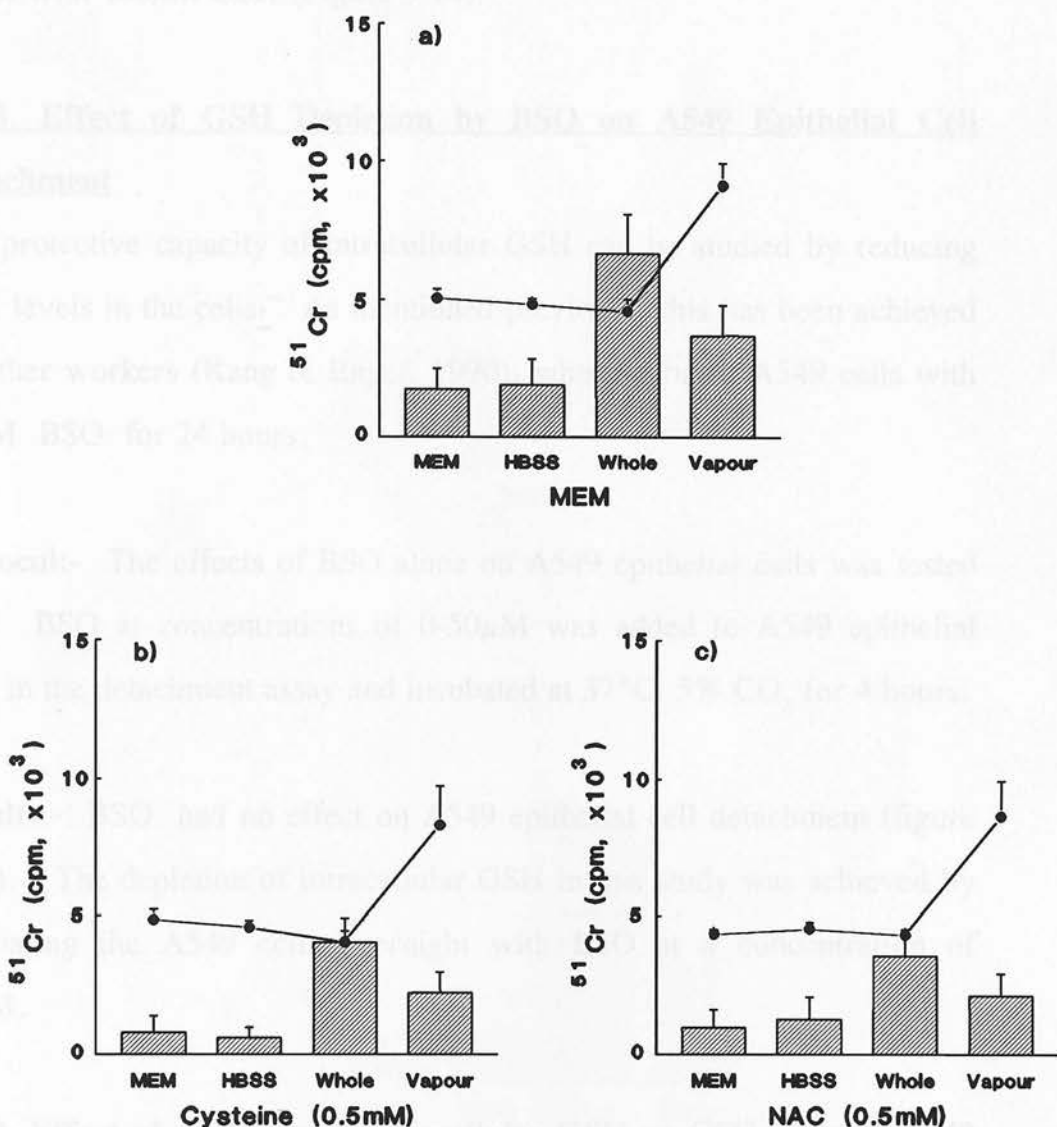


Figure 5.13. The effect of overnight pre-incubation of a) MEM, b) 0.5mM Cysteine, and c) 0.5mM NAC on the detachment (histograms) and lysis (\bullet) of A549 epithelial cells induced by 10% HBSS, whole or vapour CSC, measured in ^{51}Cr cpm. The histograms/ \bullet represent the mean of triplicate wells in four separate experiments and the bars indicate 1 SEM in each case. There were no significant changes in the detachment or lysis of cells that had been pre-incubated with either cysteine or NAC.

in cell detachment with the MEM incubated cells and those incubated in MEM with 400 μ M GSH (Figure 5.14).

5.2.8. Effect of GSH Depletion by BSO on A549 Epithelial Cell Detachment

The protective capacity of intracellular GSH can be studied by reducing GSH levels in the cells. As mentioned previously this has been achieved by other workers (Kang & Enger, 1990), who incubated A549 cells with 10 μ M BSO for 24 hours.

Protocol:- The effects of BSO alone on A549 epithelial cells was tested first. BSO at concentrations of 0-50 μ M was added to A549 epithelial cells in the detachment assay and incubated at 37°C, 5% CO₂ for 4 hours.

Results:- BSO had no effect on A549 epithelial cell detachment (figure 5.15). The depletion of intracellular GSH in this study was achieved by incubating the A549 cells overnight with BSO at a concentration of 10 μ M.

5.2.9. Effect of depletion of Intracellular GSH on CSC Treated A549 Epithelial Cells

Protocol:- The A549 epithelial cells were set up for the detachment assay as described in Section 2.2.3b. One set of plates were set up with cells suspended in CMEM, while the other set were set up with cells suspended in CMEM containing 10 μ M BSO. These were then incubated overnight at 37°C, 5% CO₂. The following morning the BSO was washed off, 200 μ l aliquots of CSC added and incubated at 37°C, 5% CO₂ for 4 hours with the A549 epithelial cells. The organic CSC and its solvent DMSO

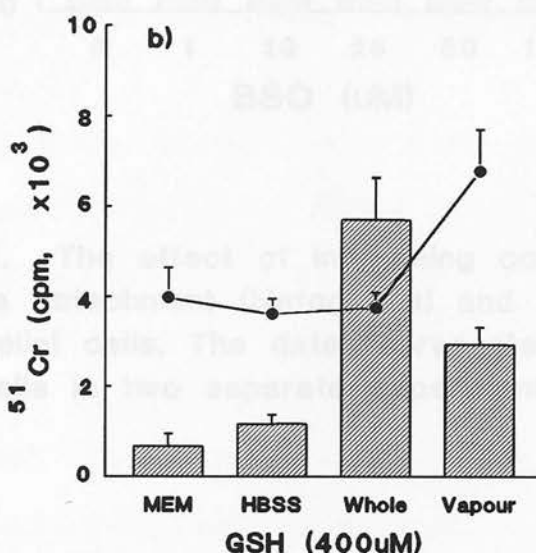
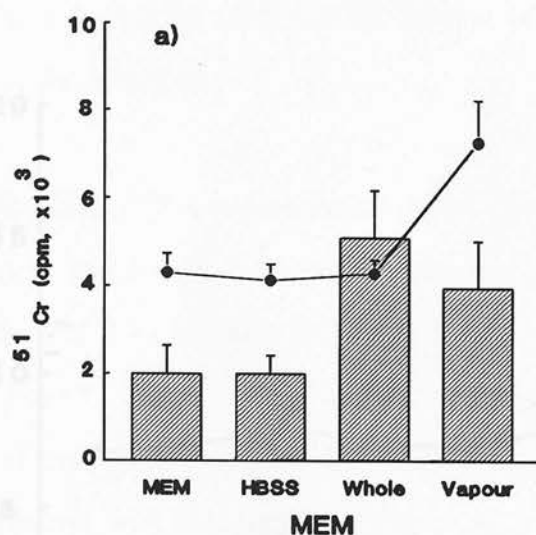


Figure 5.14. The effect of overnight pre-incubation of a) MEM, and b) 400uM GSH on the detachment (histograms) and lysis (—●—) of A549 epithelial cells induced by 10% HBSS, whole CSC or vapour CSC, measured in ^{51}Cr cpm. The histograms/—●— represent the mean of triplicate wells of four separate experiments and the bars indicate 1 SEM in each case. There were no significant changes in detachment or lysis of cells that had been pre-incubated with 400 uM GSH alone.

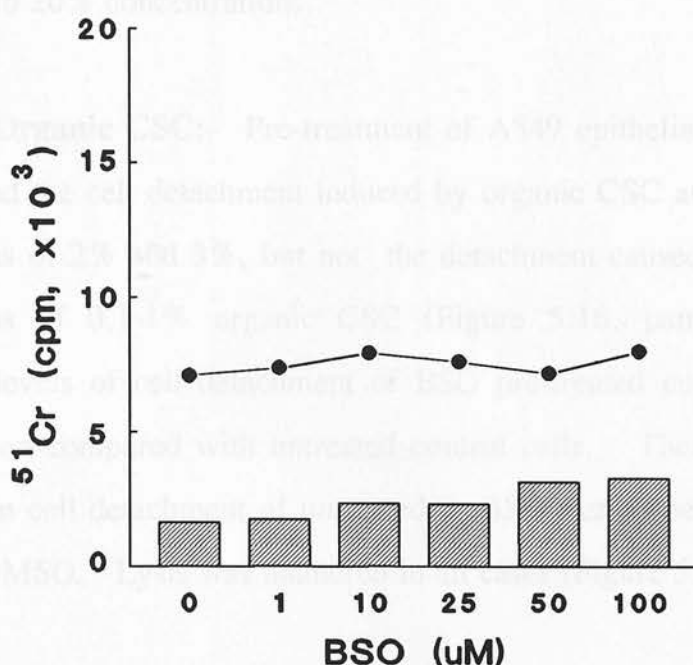


Figure 5.15. The effect of increasing concentrations of BSO on the detachment (histograms) and lysis (●) of A549 epithelial cells. The data represents the average of triplicate wells in two separate experiments.

were added to the cells at 0.1, 0.5, 1, 2 and 3% concentration, while the aqueous CSC of whole or vapour CSC and its solvent HBSS were added at 1, 5, 10 and 20% concentrations.

Results for Organic CSC:- Pre-treatment of A549 epithelial cells with BSO enhanced the cell detachment induced by organic CSC at the higher concentrations of 2% and 3%, but not the detachment caused by lower concentrations of 0.1-1% organic CSC (Figure 5.16, panel b). The background levels of cell detachment of BSO pre-treated cultures were unaltered when compared with untreated control cells. There was also no increase in cell detachment of untreated or BSO treated cells with the addition of DMSO. Lysis was unaltered in all cases (Figure 5.16).

Results for Aqueous CSC:- Pre-treatment of A549 epithelial cells with the GSH depleting agent, BSO, enhanced the cell detachment produced by all concentrations of aqueous CSC (Figure 5.17). There was increased cell lysis following exposure of BSO treated A549 cells to concentrations of 1% and 5% vapour CSC but no alteration in lysis when cells were exposed to whole CSC. The background cell detachment and cell lysis of untreated cells and BSO treated cells was the same. There was an odd result of BSO pretreated cells undergoing cell lysis when treated with only 1% whole CSC and no other concentration of whole CSC.

5.2.10. Effect of Bronchoalveolar Lavage Fluid on Aqueous CSC induced A549 Epithelial Cell Detachment

The alveolar epithelial cells are protected by the antioxidants and antiproteases present in the ELF. Preliminary experiments were carried out to investigate the protective potential of ELF against CSC induced

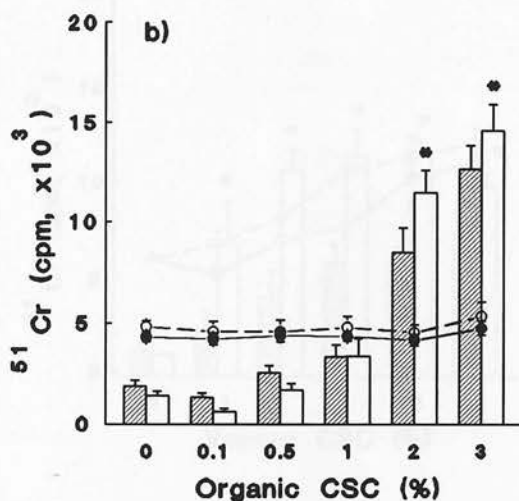
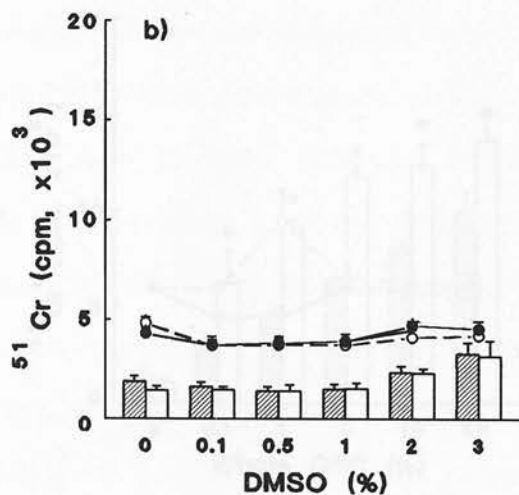


Figure 5.16. The effect of overnight pre-incubation in BSO (10uM) on the detachment (control = hatched histograms, BSO = open histograms) and lysis (control = ●, BSO = ○) of A549 epithelial cells induced by increasing concentrations of a) DMSO, and b) Organic CSC, measured in ^{51}Cr cpm. The data represents the mean of triplicate wells in five separate experiments and the bars indicate 1 SEM in each case. * $p < 0.05$ compared with the control treated with the same concentration of organic CSC.

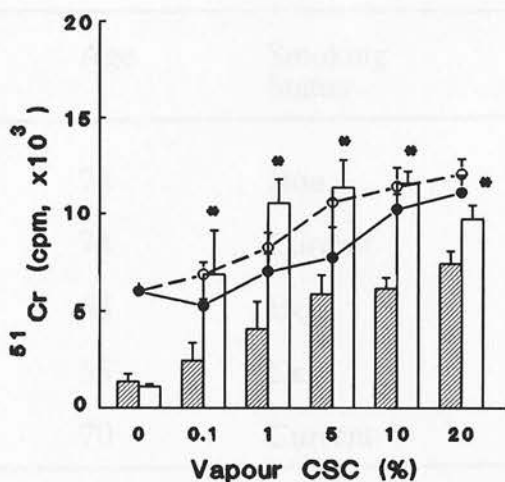
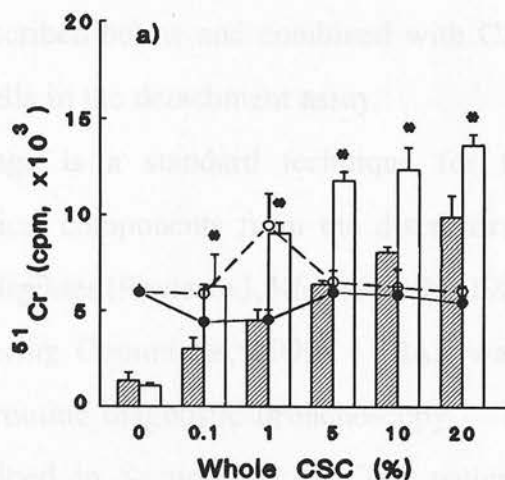


Figure 5.17. The effect of overnight pre-incubation in BSO (10uM) on the detachment (control = hatched histograms; BSO = open histograms) and lysis (control = ● ; BSO = ○) of A549 epithelial cells induced by increasing concentrations of a) whole CSC, and b) vapour CSC, measured in ^{51}Cr cpm. The data represents the mean of triplicate wells of four separate experiments and the bars indicate 1 SEM in each case. * $p < 0.05$ compared with the control treated with the same concentration of CSC.

A549 epithelial cell injury. This involved recovery of dilute ELF from the lungs of patients undergoing bronchoscopy by lavage, and this was then processed as described below and combined with CSC and added to the A549 epithelial cells in the detachment assay.

Bronchoalveolar lavage is a standard technique for the recovery of cellular and biochemical components from the distal airspace in patients with a variety of lung diseases (Reviewed, Klech & Pohl, 1989; The BAL Co-operative Group Steering Committee, 1990). BAL was obtained from patients undergoing routine diagnostic bronchoscopy. The protocol for lavage was as described in Section 2.2.2. The patient details are as follows:-

| Patient | Sex | Age | Smoking Status | CoHb |
|---------|-----|-----|----------------|------|
| 1 | F | 78 | Non | 1.65 |
| 2 | F | 74 | Current | 3.8 |
| 3 | M | 68 | Ex | 2.8 |
| 4 | M | 55 | Ex | 1.6 |
| 5 | M | 70 | Current | 7.5 |

BALF obtained in this way was tested for its protective potential against CSC induced cell injury.

Protocol:- Aqueous CSC either whole or vapour CSC (10%) was combined with BALF (5,10 or 50%) or saline controls (5,10 or 50% dilutions) in MEM (total 200 μ l aliquots) and incubated with the A549 epithelial cells in the detachment assay for 4 hours at 37°C, 5% CO₂.

Results:- Neither saline nor BALF alone had any adverse effect on the

cells. Aqueous CSC alone produced the expected increase in cell detachment and cell lysis. The addition of BALF with either whole or vapour CSC did not significantly protect the cells against injury (Figure 5.19), in combining saline with the aqueous CSC had no effect (Figure 5.18).

5.3. [³H] THYMIDINE ASSAY

The antioxidants of SOD ($50\mu\text{g}.\text{ml}^{-1}$), catalase ($50\mu\text{g}.\text{ml}^{-1}$) and GSH (200-400 μM) and antiproteinase α -1PI, ($50\mu\text{g}.\text{ml}^{-1}$) at standard concentrations were added into the [³H] thymidine assay (Section 2.2.3d.) at the same time as the CSC to A549 epithelial cells, which were incubated overnight at 37°C, 5% CO₂ and then harvested the following morning. The organic CSC was used at a 2% concentration and whole and vapour CSC at a 10% dilution.

Results for Organic CSC:- The suppression of [³H] uptake by A549 epithelial cells by organic CSC was not affected by the addition of any of the antioxidants or by the addition of the antiproteinase (Figures 5.20 & 5.21).

Results for Aqueous CSC:- There was no increase in the uptake of [³H] thymidine when the CSC was combined with SOD, catalase or α -1PI, compared with CSC alone (Figure 5.22). Only GSH protected against the reduction in [³H] uptake by A549 epithelial cells treated with either whole or vapour CSC (Figure 5.23).

SUMMARY

Of the limited number of antioxidants examined, only GSH conferred

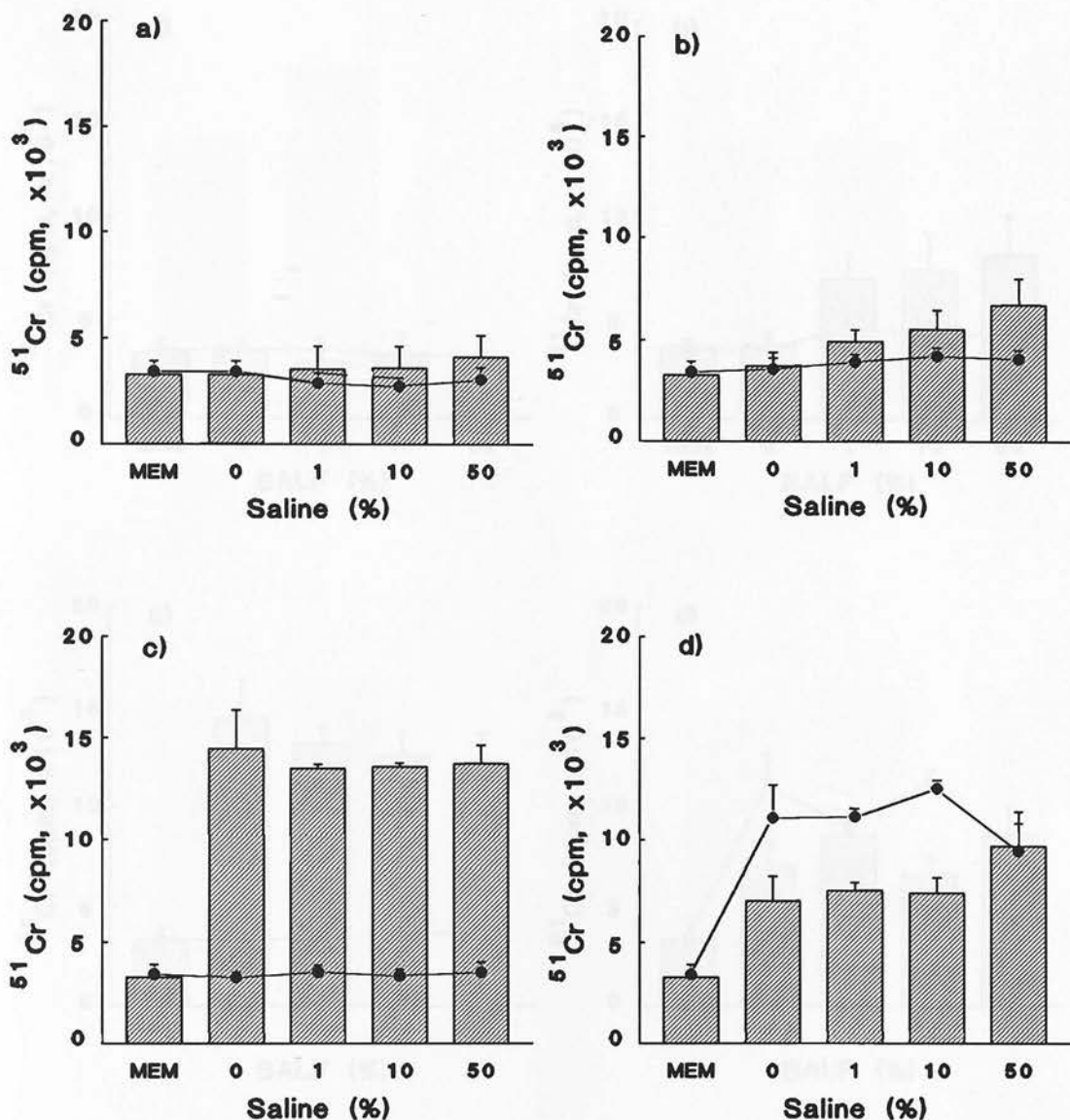


Figure 5.18. The effect of increasing concentrations of saline on a) MEM, b) HBSS, c) 10% whole CSC, and d) 10% vapour CSC on detachment (histograms) and lysis (●) of A549 epithelial cells, measured in ^{51}Cr cpm. The histograms /-● represent the mean of triplicate wells in five separate experiments and the bars indicate 1 SEM in each case.

There was no significant change in detachment or lysis with the addition of saline to each treatment.

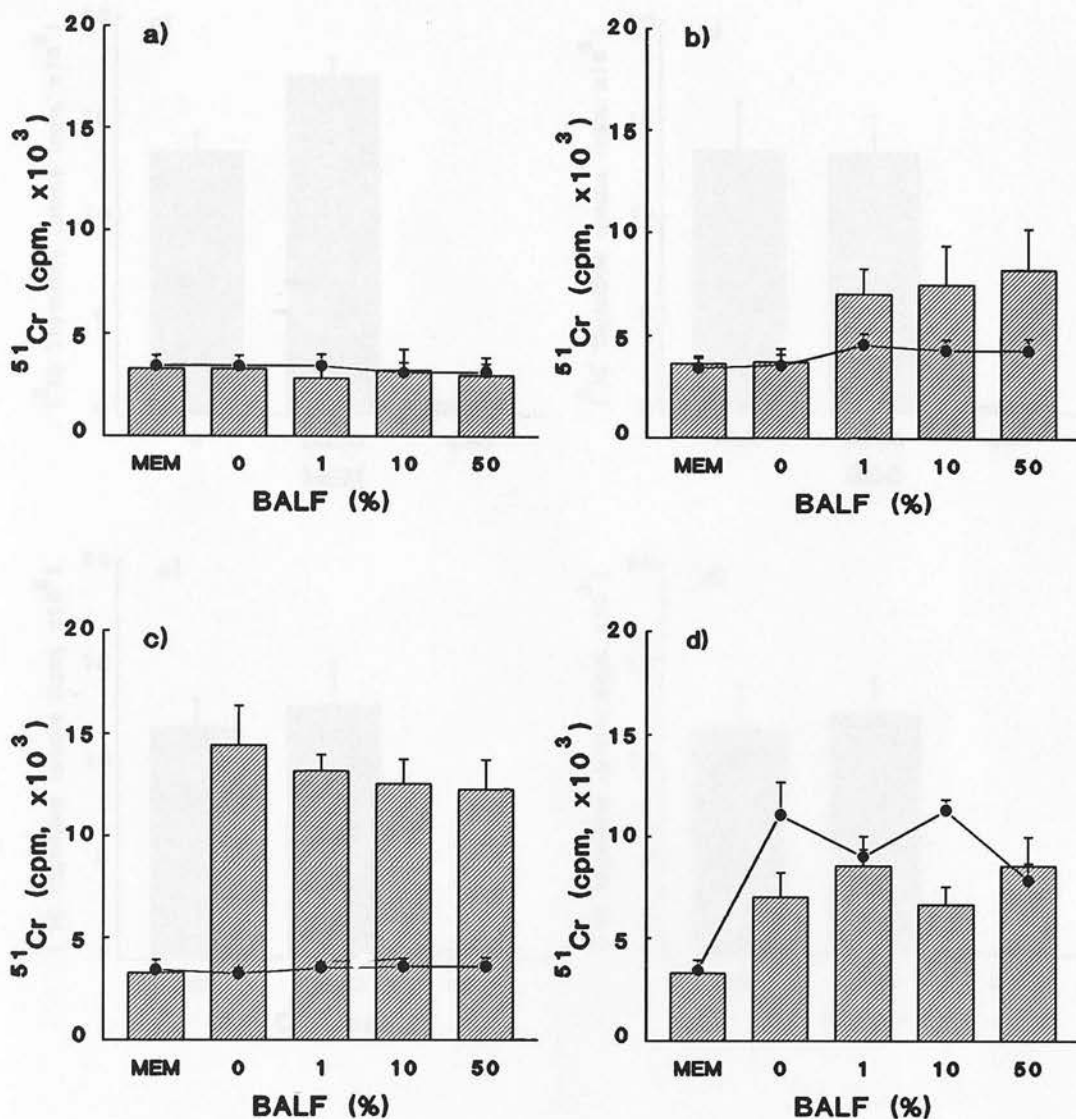


Figure 5.19. The effect of increasing concentration of BALF on A549 epithelial cell detachment (histograms) and lysis (—●—) induced by a) MEM, b) 10% HBSS, c) 10% whole CSC, and d) 10% vapour CSC, measured in ^{51}Cr cpm. The histograms/—●— represent the mean of triplicate wells in five separate experiments and the bars indicate 1 SEM in each case. There was no significant change in detachment or lysis with the addition of BALF for each treatment.

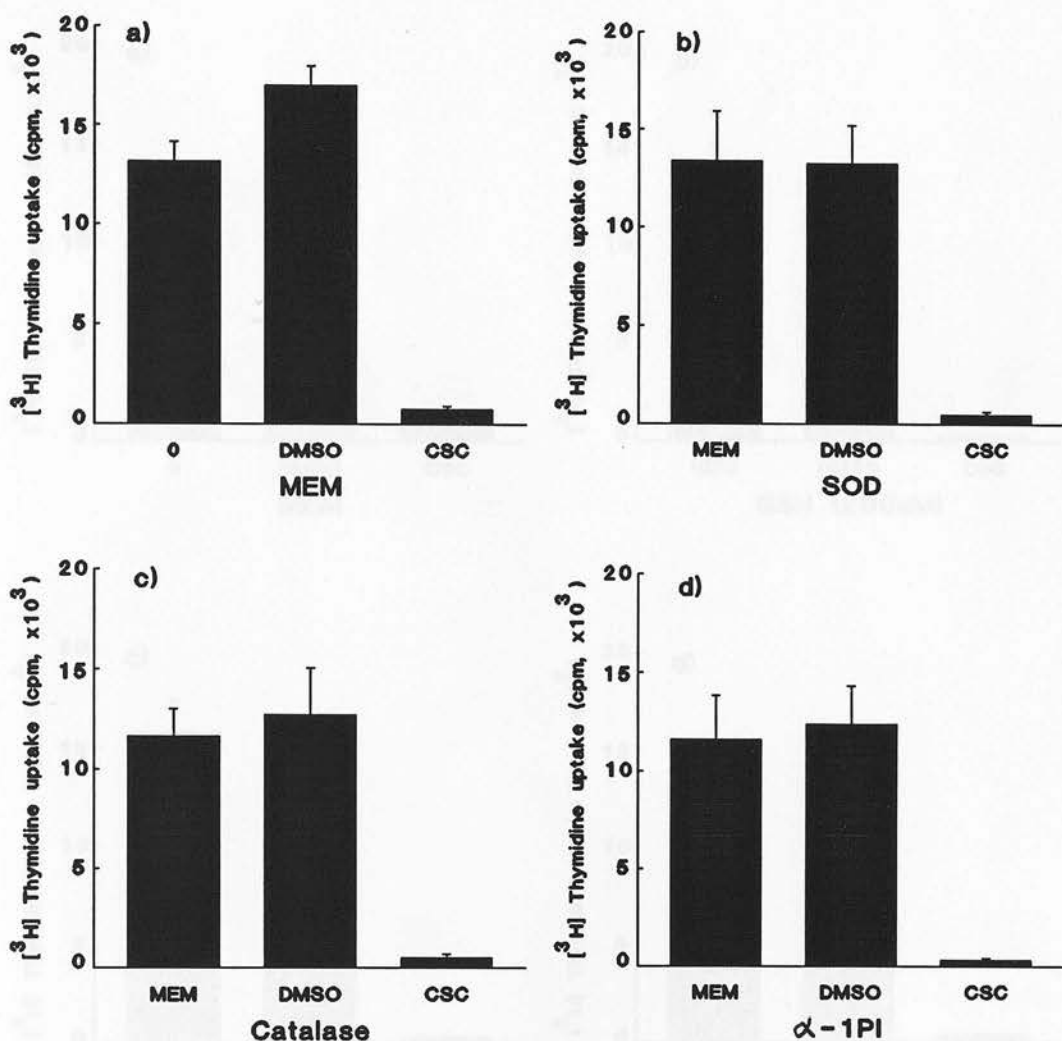


Figure 5.20. The effect of addition of a) MEM, b) SOD, c) catalase, and d) α -1PI on the $[^3\text{H}]$ thymidine uptake of A549 epithelial cells treated with 2% organic CSC, measured in $[^3\text{H}]$ cpm. The histograms represent the mean of triplicate wells in three separate experiments and the bars indicate 1 SEM in each case. There was no significant change in the $[^3\text{H}]$ thymidine uptake with the addition of any of the treatments.

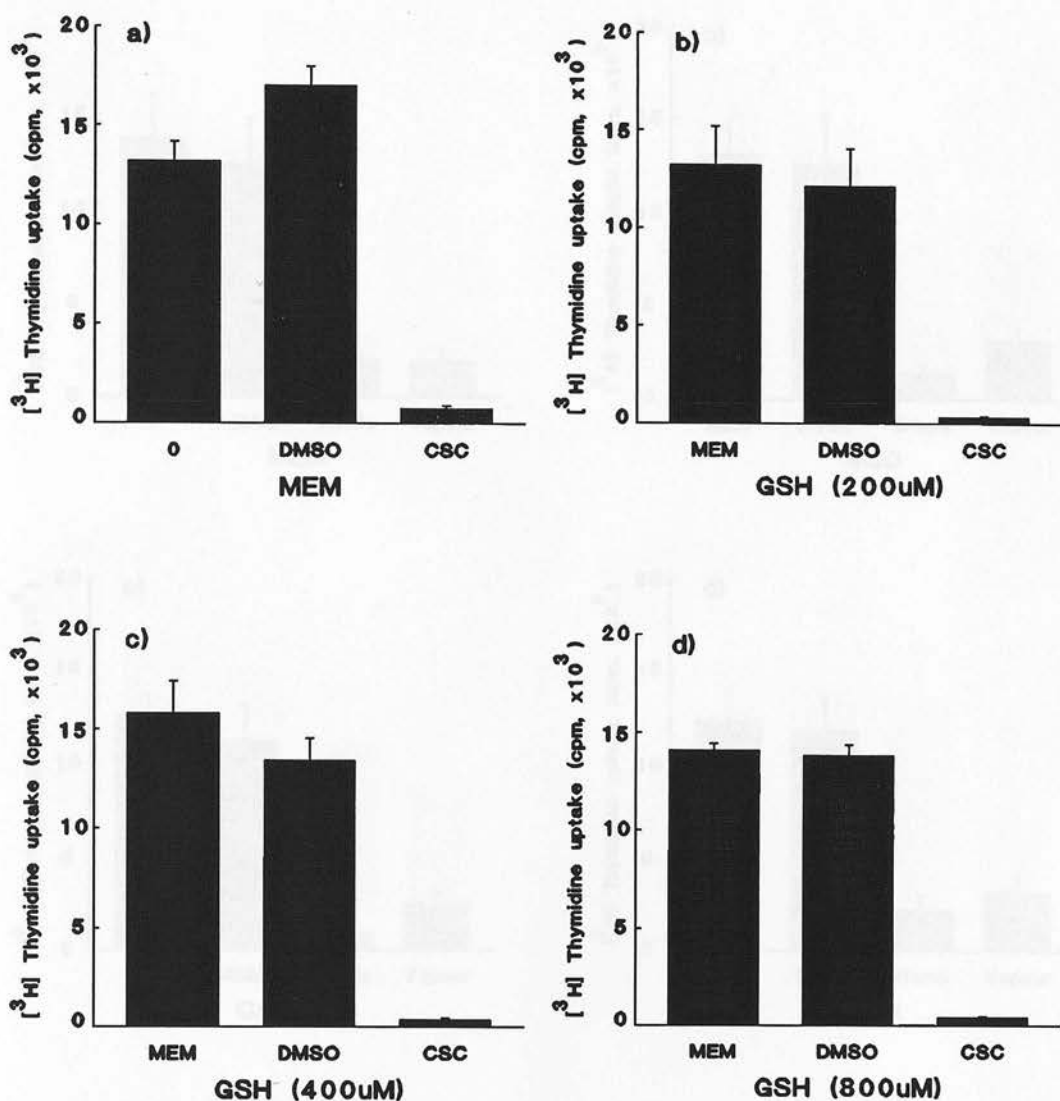


Figure 5.21. The effect of addition of a) MEM, b) 200uM GSH, c) 400uM GSH, and d) 800uM GSH on the $[^3\text{H}]$ thymidine uptake of A549 epithelial cells treated with 2% organic CSC, measured in $[^3\text{H}]$ cpm. The histograms represent the mean of triplicate wells in three separate experiments and the bars indicate 1 SEM in each case. There was no significant change in $[^3\text{H}]$ thymidine uptake with the addition of any of the treatments.

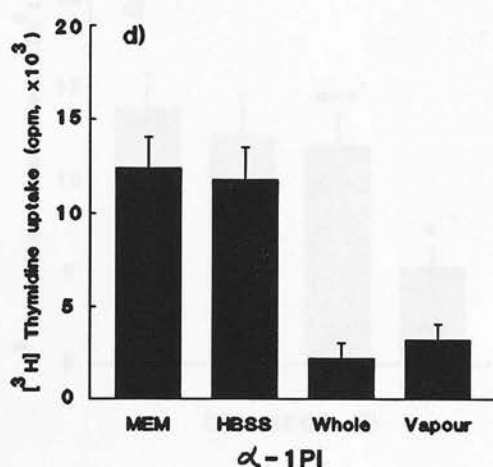
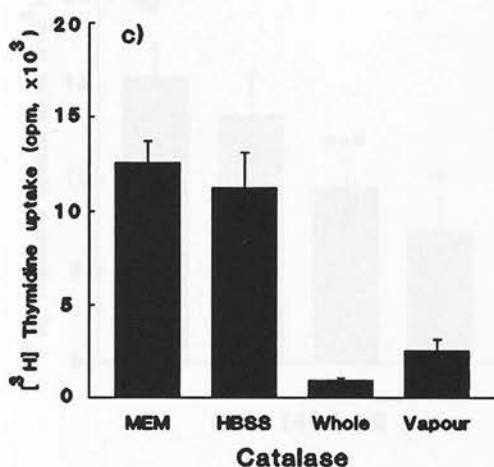
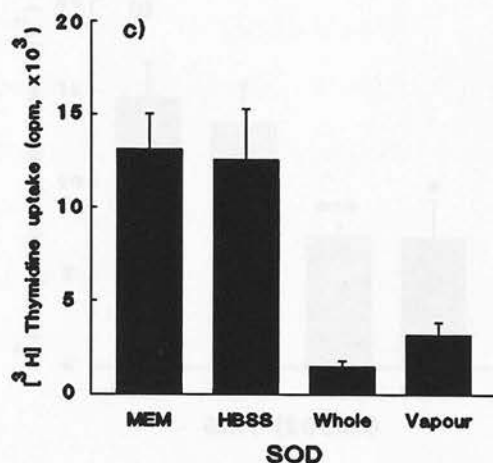
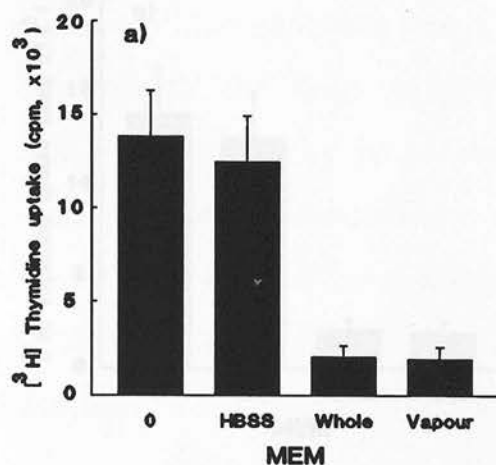


Figure 5.22. The effect of addition of a) MEM, b) SOD, c) catalase, and d) α -1PI on the [³H] thymidine uptake of A549 epithelial cells treated with 10% HBSS, whole or vapour CSC, measured in [³H] cpm. The histograms represent the mean of triplicate wells in four separate experiments and the bars indicate 1 SEM in each case. There was no significant change in [³H] thymidine uptake with the addition of any of the treatments.

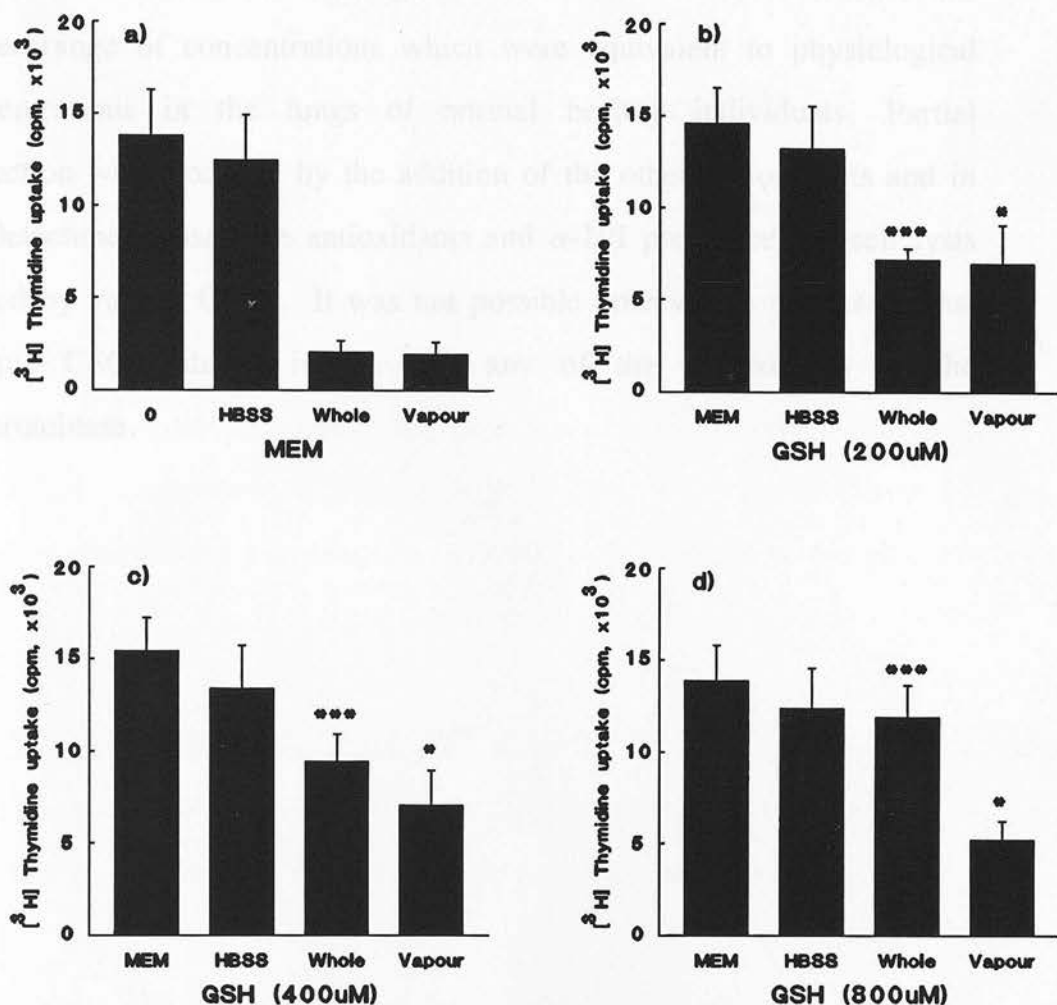


Figure 5.23. The effect of addition of a) MEM, b) 200uM GSH, c) 400uM GSH, and d) 800uM GSH on the [³H] thymidine uptake of A549 epithelial cells treated with 10% HBSS, whole or vapour CSC, measured in [³H] cpm. The histograms represent the mean of triplicate wells in four separate experiments and the bars indicate 1 SEM in each case. * p < 0.05; *** p < 0.001 compared with the CSC alone.

the greatest protection of A549 epithelial cells against cigarette smoke or aqueous CSC induced cell damage. However, this only occurred at the higher range of concentrations which were equivalent to physiological concentrations in the lungs of normal healthy individuals. Partial protection was produced by the addition of the other antioxidants and in the detachment assay the antioxidants and α -1PI prevented the cell lysis caused by vapour CSC. It was not possible however to protect against organic CSC induced injury with any of the antioxidants or the antiprotease.

The ECM components examined in this work were types I and IV collagen, fibronectin and laminin, which are all present at the alveolar level.

6.1. A549 EPITHELIAL CELL ATTACHMENT

The influence of ECM components on the attachment ability of control or cigarette smoke-exposed A549 epithelial cells was examined.

Protocol: The A549 epithelial cells were trypsinised off the culture flasks and ^{51}Cr labelled as described in Section 2.2.3a. These cells were resuspended in MEM (1×10^6 cells ml $^{-1}$) and exposed to either 4 puffs of whole or vapour phase cigarette smoke or sham exposed in air (Section 2.2.4). The cells were immediately washed in MEM (200 μ l, 5 min), and then resuspended in CMEM at a concentration of 1×10^6 cells ml $^{-1}$. Aliquots (200 μ l) of each of the cell suspensions were then added in triplicate to the plates coated with ECM components (Section 2.2.6.) and the cells were incubated at 37°C, 5% CO $_2$ for up to 4 hours. At the time points of 1, 2 and 4 hours triplicate wells of each condition on each plate were harvested and the cell associated cpm measured (Section 2.2.3a).

CHAPTER 6

EXTRACELLULAR MATRIX COMPONENTS

As discussed in the Introduction the extracellular matrix components are important in the development and repair, as well as the structure of the lung. This Chapter investigates the role of certain ECM components in A549 epithelial cell attachment and detachment with reference to C.S.C. The ECM components examined in this work were types I and IV collagen, fibronectin and laminin, which are all present at the alveolar level.

6.1. A549 EPITHELIAL CELL ATTACHMENT

The influence of ECM components on the attachment ability of control or cigarette smoke-exposed A549 epithelial cells was examined.

Protocol:- The A549 epithelial cells were trypsinized off the culture flasks and ^{51}Cr labelled as described in Section 2.2.3a. These cells were resuspended in MEM (1×10^6 cells ml^{-1}) and exposed to either 4 puffs of whole or vapour phase cigarette smoke or sham exposed in air (Section 2.2.4.). The cells were immediately washed in MEM (200g, 5 minutes) and then resuspended in CMEM at a concentration of $\times 10$ cells ml^{-1} . Aliquots (200 μl) of each of the cell suspensions were then added in triplicate to the plates coated with ECM components (Section 2.2.6.) and the cells were incubated at 37°C , 5% CO_2 for up to 4 hours. At the time points of 1,2 and 4 hours triplicate wells of each condition on each plate were harvested and the cell associated cpm measured (Section 2.2.3a).

Results:- The level of attachment was very variable but each experiment followed the same trends. There was a differing rate of attachment depending on the ECM component. At 1 hour the percentage of attachment of sham exposed cells to collagen I and collagen IV was much greater than that attached to the control culture plastic plates, and this was also the case at the 2 and 4 hour time point. The percentage of attachment of the whole (Figure 6.1, panel c) and vapour (Figure 6.1, panel d) cigarette smoke exposed cells was greatly reduced compared with the untreated or the sham exposed cells. There was greater a number of these smoke exposed cells attached to collagen I and IV than any of the other ECM coated plates or the culture plastic plate.

6.2. A549 Epithelial Cell Detachment

The series of detachment assays involved precoating the 96-well plates with the ECM components. As well as the aforementioned glycoprotein, the experiment also contained plates precoated with Bovine serum albumin (BSA) to act as a non-specific binding control.

6.2.1. Organic CSC

Protocol:- This condensate was used at 2% dilution with 4 hour incubation at 37°C and 5% CO₂. Triplicate aliquots of the organic CSC were added to A549 epithelial cells which had been seeded in the normal way (Section 2.2.3b.) on to 96-well plates precoated with ECM components (Section 2.2.6.).

Results:- At this concentration there was no observed increase in cell lysis with any treatment on any of the plates. There was increased detachment of condensate-treated A549 epithelial cells plated on untreated

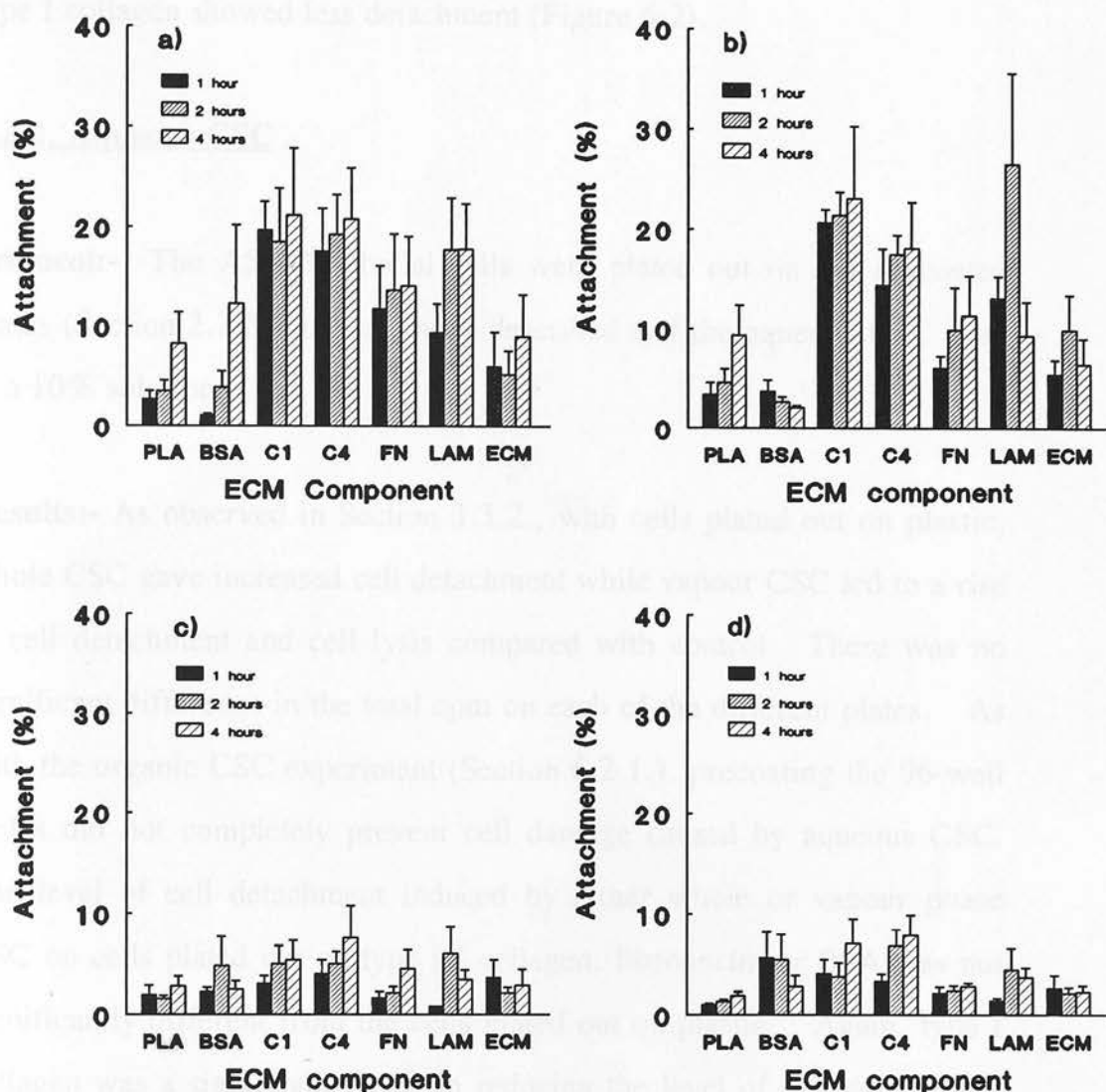


Figure 6.1. The effect of pre-coating tissue culture plates with ECM components on the attachment of A549 epithelial cells over time after exposure to 4 puffs of cigarette smoke, a) control, b) sham, c) whole, and d) vapour, measured in percentage of total cells plated out. The histograms represent the mean of triplicate wells in four separate experiments and the bars indicate 1 SEM in each case.

96-well culture plates. This level of organic CSC induced detachment was constant in all but one of the pre-coated plates, and cells plated out on type I collagen showed less detachment (Figure 6.2).

6.2.2. Aqueous CSC

Protocol:- The A549 epithelial cells were plated out on the precoated plates (Section 2.2.6.) as previously described and the aqueous CSC used at a 10% solution.

Results:- As observed in Section 3.3.2., with cells plated out on plastic, whole CSC gave increased cell detachment while vapour CSC led to a rise in cell detachment and cell lysis compared with control. There was no significant difference in the total cpm on each of the different plates. As with the organic CSC experiment (Section 6.2.1.), precoating the 96-well plates did not completely prevent cell damage caused by aqueous CSC. The level of cell detachment induced by either whole or vapour phase CSC on cells plated out on type IV collagen, fibronectin or BSA was not significantly different from the cells plated out on plastic. Again, type I collagen was a significant factor in reducing the level of cell detachment induced by whole CSC (Figure 6.3, panel c) or vapour CSC (Figure 6.3, panel d). There was also significantly less lysis of A549 epithelial cells plated out on collagen I when compared with cells plated out on culture plastic.

SUMMARY

The ECM components influenced the rate of attachment of control and cigarette smoke exposed A549 epithelial cells although the level of

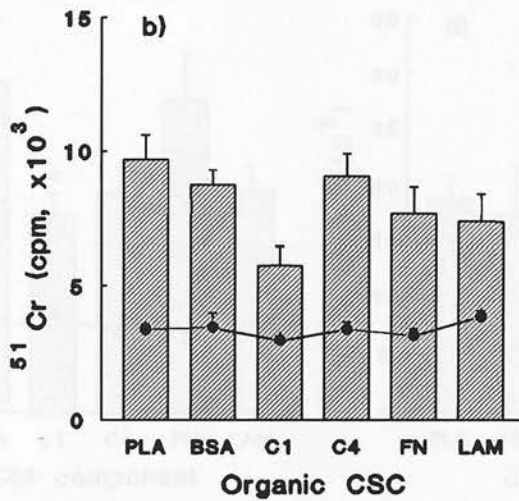
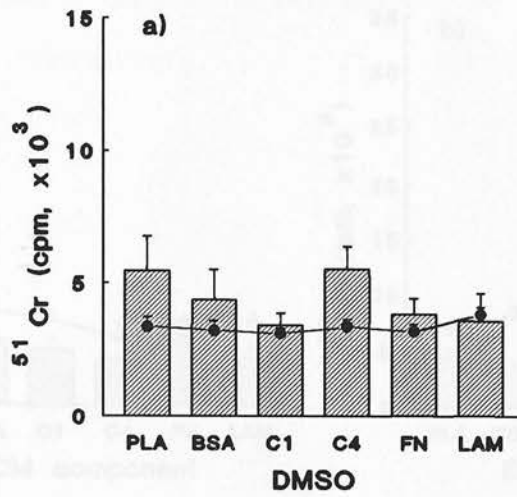


Figure 6.2. The effect of pre-coating tissue culture plates with ECM components on the detachment (histograms) and lysis (\bullet) of A549 epithelial cells induced by a) 2% DMSO , and b) 2% organic CSC, measured in ^{51}Cr cpm. The histograms/ \bullet represent the mean of triplicate wells in three separate experiments and the bars indicate 1 SEM in each case.

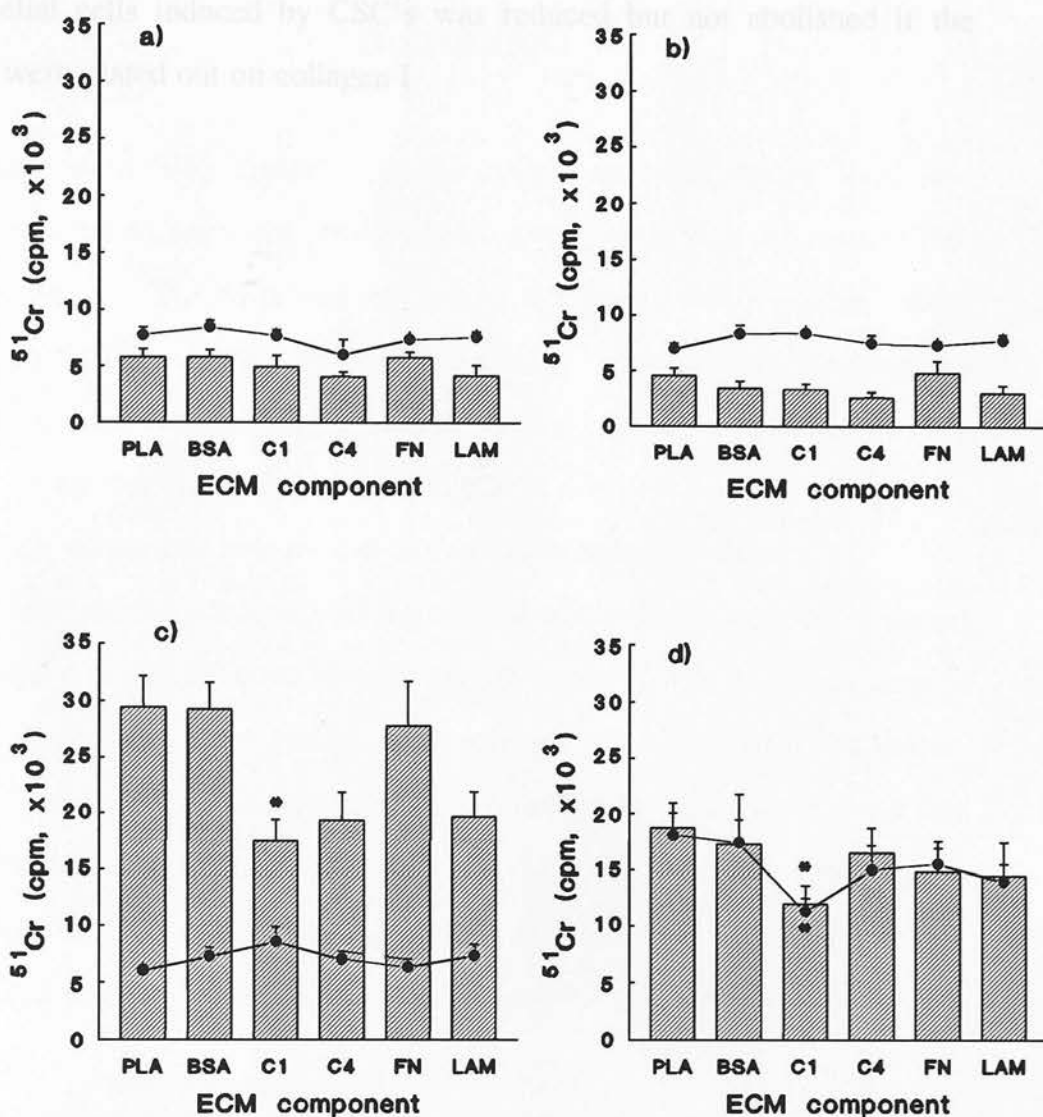


Figure 6.3. The effect of pre-coating tissue culture plates with ECM components on the detachment (histograms) and lysis (—●—) of A549 epithelial cells induced by a) MEM, b) 10% HBSS, c) 10% whole CSC, c) 10% vapour CSC, measured in ^{51}Cr cpm. The histograms/—●— represent the mean of triplicate wells in five separate experiments and the bars indicate 1 SEM in each case. * $p < 0.05$ when compared with cells plated out on plastic for that treatment.

attachment was still reduced with the cigarette smoke exposed cells compared with control cells. The detachment and lysis of the A549 epithelial cells induced by CSC's was reduced but not abolished if the cells were plated out on collagen I.

To understand why cigarette smoke causes epithelial injury it is first necessary to examine the basic factors involved in cell attachment and detachment. The following studies in this thesis will examine three possible factors: (1) the cell-matrix adhesion molecule, i.e. the integrins which will be expanded upon in this Chapter, (2) the cytoskeleton, with a focus being the main focus of Chapter 3 and (3) cell signalling with emphasis on protein kinases and calcium followed in Chapter 4.

To investigate the possibility of cigarette smoke or CSC-induced epithelial cell injury being mediated through the integrins, it was first necessary to elucidate the role of integrins in epithelial cell attachment and detachment. After this was established then the question of changes in the expression of epithelial cells with exposure to cigarette smoke was addressed. A preliminary study was also carried out to relate the epithelial cell line to the human type II pneumocyte in relation to integrin expression.

Two epithelial cell lines, A549 and H1299 were studied in the following series of experiments. It was not possible to use the R12C, as monoclonal antibodies (mAb's) required for certain experiments were specific to human cells and did not cross-react with rat cells.

1. Effect of Calcium Chelation on Epithelial Cell Detachment

As outlined in Chapter 1, divalent cations are vital for the functional activity of the integrins. Experimentally it is possible to remove the cells from the extracellular environment using the chelators

CHAPTER 7

INTEGRINS

To understand why cigarette smoke causes epithelial injury it is first necessary to examine the basic factors involved in cell attachment and detachment. The following studies in this thesis will examine three possible factors (1) the cell-surface adhesion molecule i.e. the integrins which will be expanded upon in this Chapter, (2) the cytoskeleton, with actin being the main focus of Chapter 8 and (3) cell signalling with emphasis on protein kinases and calcium focussed in Chapter 9.

To investigate the possibility of cigarette smoke or CSC-induced epithelial cell injury being mediated through the integrins, it was first necessary to elucidate the role of integrins in epithelial cell attachment and detachment. After this was established then the question of changes in integrin expression of epithelial cells with exposure to cigarette smoke was addressed. A preliminary study was also carried out to relate the epithelial cell line to the human type II pneumocyte in relation to integrin profile.

These epithelial cell lines A549 and BEA-2B were studied in the following series of experiments. It was not possible to use the RTTC, as the monoclonal antibodies (mAb's) required for certain experiments were specific to human cells and did not cross-react with rat cells.

7.1. Effect of Cation Chelation on Epithelial Cell Detachment

As mentioned in Chapter 1, divalent cations are vital for the functional integrity of the integrins. Experimentally it is possible to remove the cations from the extracellular environment using the chelators,

ethylenediaminetetraacetic acid (EDTA) and ethyleneglycoltetraacetic acid (EGTA).

Protocol:- A549 and BEA-2B cells were subjected to increasing concentrations (10, 20, 50, 100, 200 and 500 μ M and 1mM) of both EDTA and EGTA dissolved in MEM in the detachment assay for the standard 4 hours incubation at 37°C, 5% CO₂.

Results for A549 Epithelial Cells:- There was an increase in A549 epithelial cell detachment with solutions of $\geq 20 \mu$ M EDTA in MEM, and cell lysis did not rise significantly above baseline values until a concentration of 500 μ M EDTA (Figure 7.1, panel a). Maximal detachment with minimal lysis was reached at 50 μ M EDTA. Detachment of A549 epithelial cells occurred at concentrations of EGTA $\geq 10 \mu$ M (Figure 7.1, panel b) and followed a similar pattern of A549 epithelial cell detachment and lysis as cells treated with EDTA, although detachment was still predominant at 200 μ M EGTA and lysis was not significant until the cells were treated with 500 μ M EGTA.

Results for BEA-2B Epithelial Cells:- The profiles of cell detachment and lysis for BEA-2B cells treated with either EDTA or EGTA was similar (Figure 7.2). There was maximal detachment of the BEA-2B epithelial cells treated with either 200 μ M EDTA or EGTA. Detachment occurred earlier with 50 μ M EDTA but not with cells treated with 50 μ M EGTA. Cell lysis became the predominant feature at 500 μ M in both cases. This was also a similar pattern to that seen with the A549 epithelial cells.

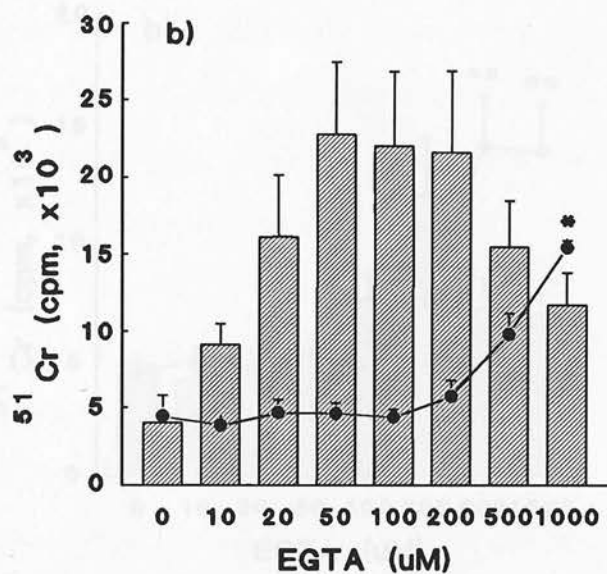
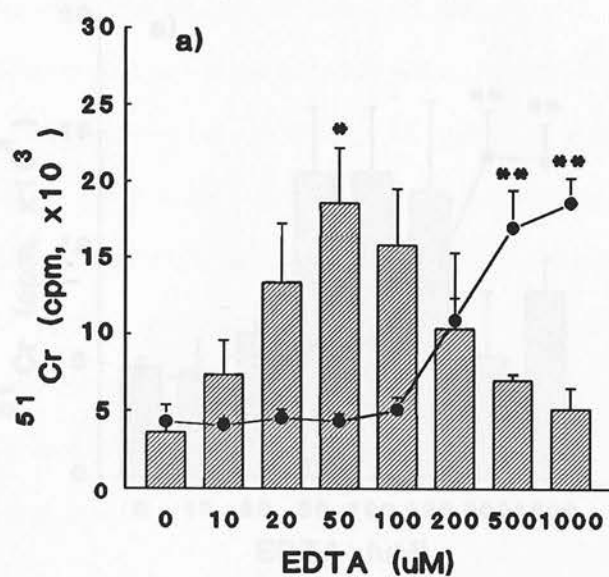


Figure 7.1. The effect of increasing concentrations of a) EDTA, and b) EGTA on the detachment (histograms) and lysis (\bullet) of A549 epithelial cells, measured in ^{51}Cr cpm. The histograms/ \bullet represent the mean of triplicate wells of six separate experiments and the bars indicate 1 SEM in each case. * $p < 0.05$; ** $p < 0.01$ compared with medium alone (0).

7.2. Effect of the Tripeptide, *Asparto-Glutamic-Aspartic Acid*, On Epithelial Detachment

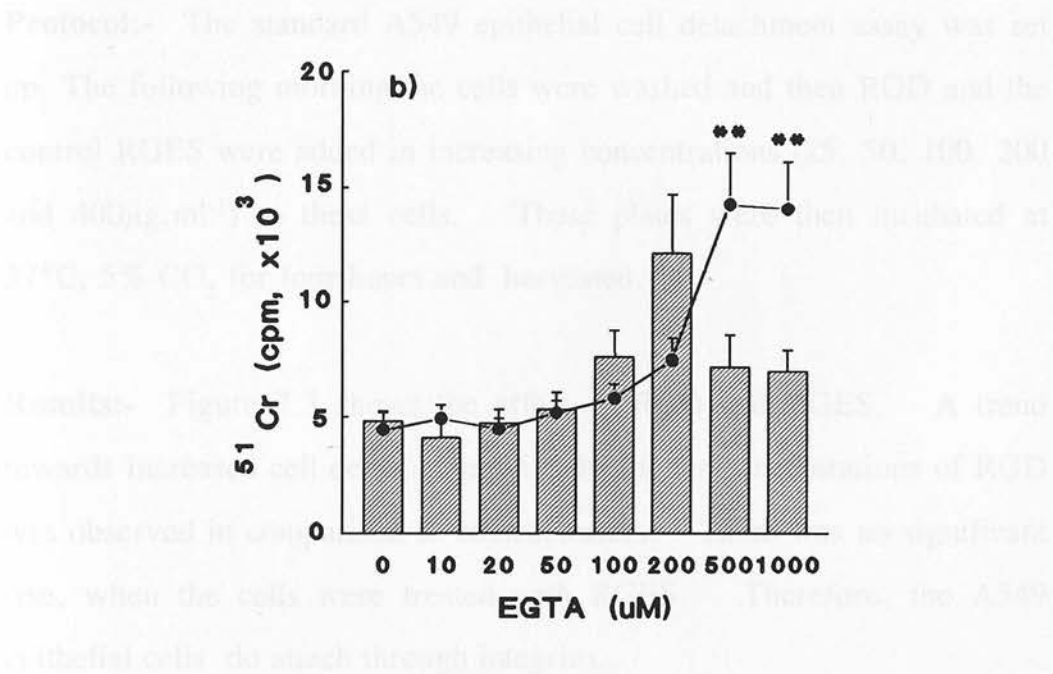
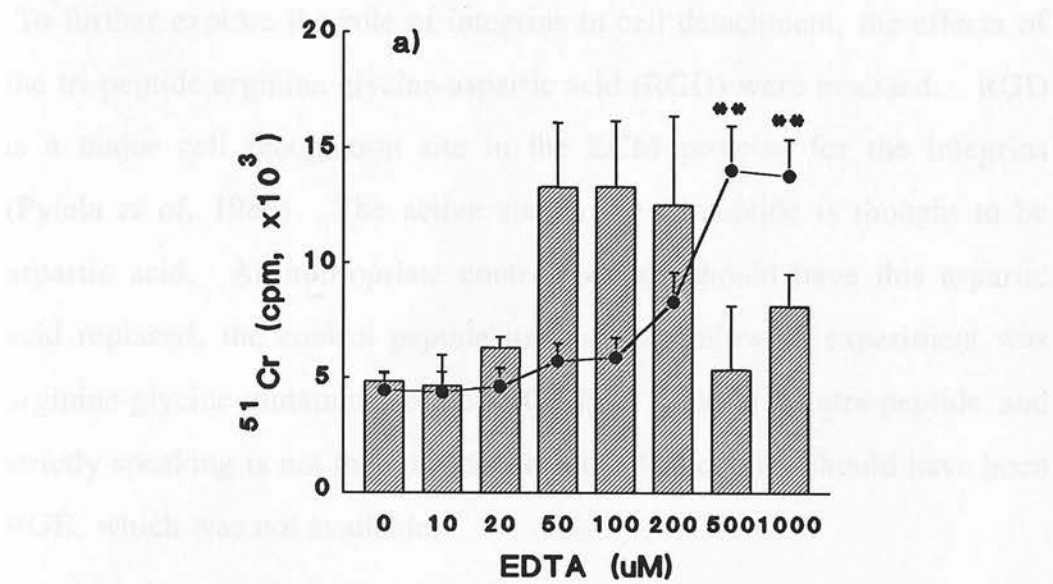


Figure 7.2. The effect of increasing concentrations of a) EDTA, and b) EGTA on the detachment (histograms) and lysis (\bullet) of BEA-2B epithelial cells, measured in ^{51}Cr cpm. The histograms/ \bullet represent the mean of triplicate wells and the bars indicate 1 SEM in each case. ** p < 0.01 compared with medium alone (0).

7.2. Effect of the Tri-peptide Arginine-Glycine-Aspartic Acid On Epithelial Detachment

To further explore the role of integrins in cell detachment, the effects of the tri-peptide arginine-glycine-aspartic acid (RGD) were assessed. RGD is a major cell recognition site in the ECM proteins for the integrins (Pytela *et al*, 1986). The active site in the tri-peptide is thought to be aspartic acid. An appropriate control peptide should have this aspartic acid replaced, the control peptide used in the following experiment was arginine-glycine-glutamine-serine (RGES). This is a tetra-peptide and strictly speaking is not the correct control. The control should have been RGE, which was not available.

Protocol:- The standard A549 epithelial cell detachment assay was set up. The following morning the cells were washed and then RGD and the control RGES were added in increasing concentrations (25, 50, 100, 200 and 400 $\mu\text{g}.\text{ml}^{-1}$) to these cells. These plates were then incubated at 37°C, 5% CO₂ for four hours and harvested.

Results:- Figure 7.3 shows the effect of RGD and RGES. A trend towards increased cell detachment with the higher concentrations of RGD was observed in comparison to control values. There was no significant rise, when the cells were treated with RGES. Therefore, the A549 epithelial cells do attach through integrins.

7.3. Characterisation of ECM Integrins on Epithelial Cells

Then next step was to find out which integrins were expressed by the two epithelial cell lines and which were important in mediating cell attachment. This was done by firstly partially characterising both

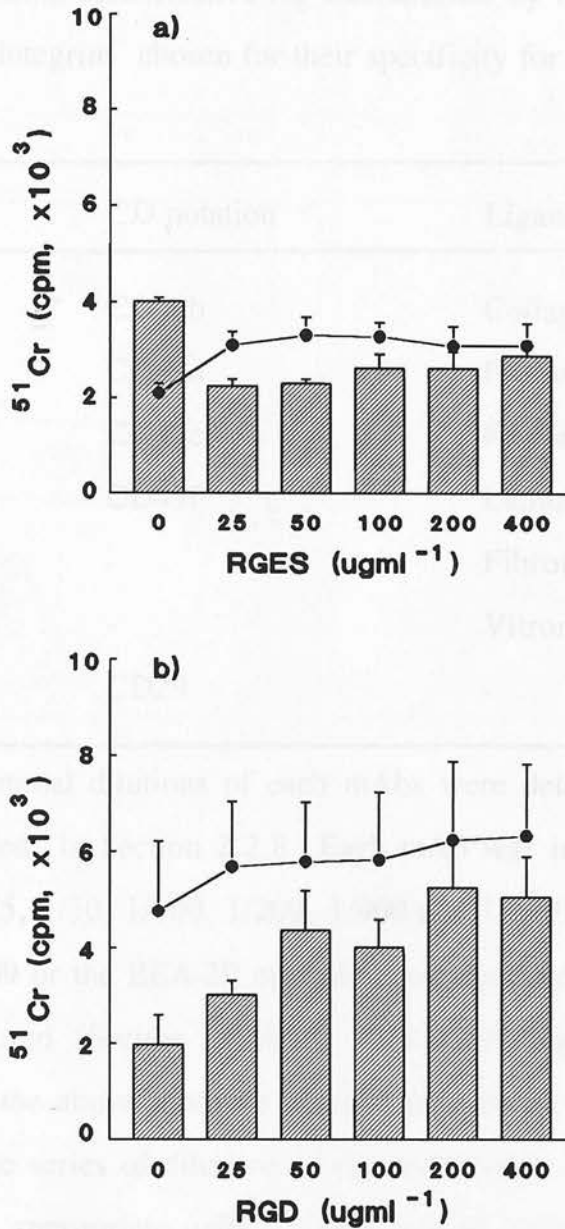


Figure 7.3. The effect of a) RGES, and b) RGD on the detachment (histograms) and lysis (\bullet) of A549 epithelial cells measured in ^{51}Cr cpm. The histograms/ \bullet represent the mean of triplicate wells of five separate experiments and the bars indicate 1 SEM in each case. The trend is towards increased detachment with RGD.

epithelial cell lines for ECM integrins using a range of mAbs to these integrins and measuring their relative log fluorescence by flow cytometry (Section 2). The integrins chosen for their specificity for ECM proteins were as follows.

| Subunit | CD notation | Ligands |
|-------------------|-------------|-------------------------|
| α_2 | CD49b | Collagen/Laminin |
| α_4 | CD49d | Fibronectin (GSII site) |
| α_5 | CD49e | Fibronectin (RGD site) |
| α_6 | CD49f | Laminin |
| α_v | - | Fibronectin/vitronectin |
| $\alpha_v\beta_3$ | - | Vitronectin |
| β_1 | CD29 | - |

Protocol:- The optimal dilutions of each mAbs were determined using the method described in Section 2.2.8 Each mAb was initially diluted over a range of 1/25, 1/50, 1/100, 1/200, 1/400 and 1/800 and incubated with either the A549 or the BEA-2B epithelial cells and their relative log fluorescence measured (Section 2.2.8). Once the highest level of fluorescence using the above dilutions was pin-pointed for each mAb, a second more precise series of dilutions of the mAb were made and then incubated with the appropriate cells. This second series of dilutions established the level at which the mAbs were used in subsequent experiments. A control set of both types of cells were also incubated with wash buffer alone and no mAb and treated with the second layer of either anti-mouse FITC or anti-rat FITC; the reason being to check for non-specific binding of the second layer antibody.

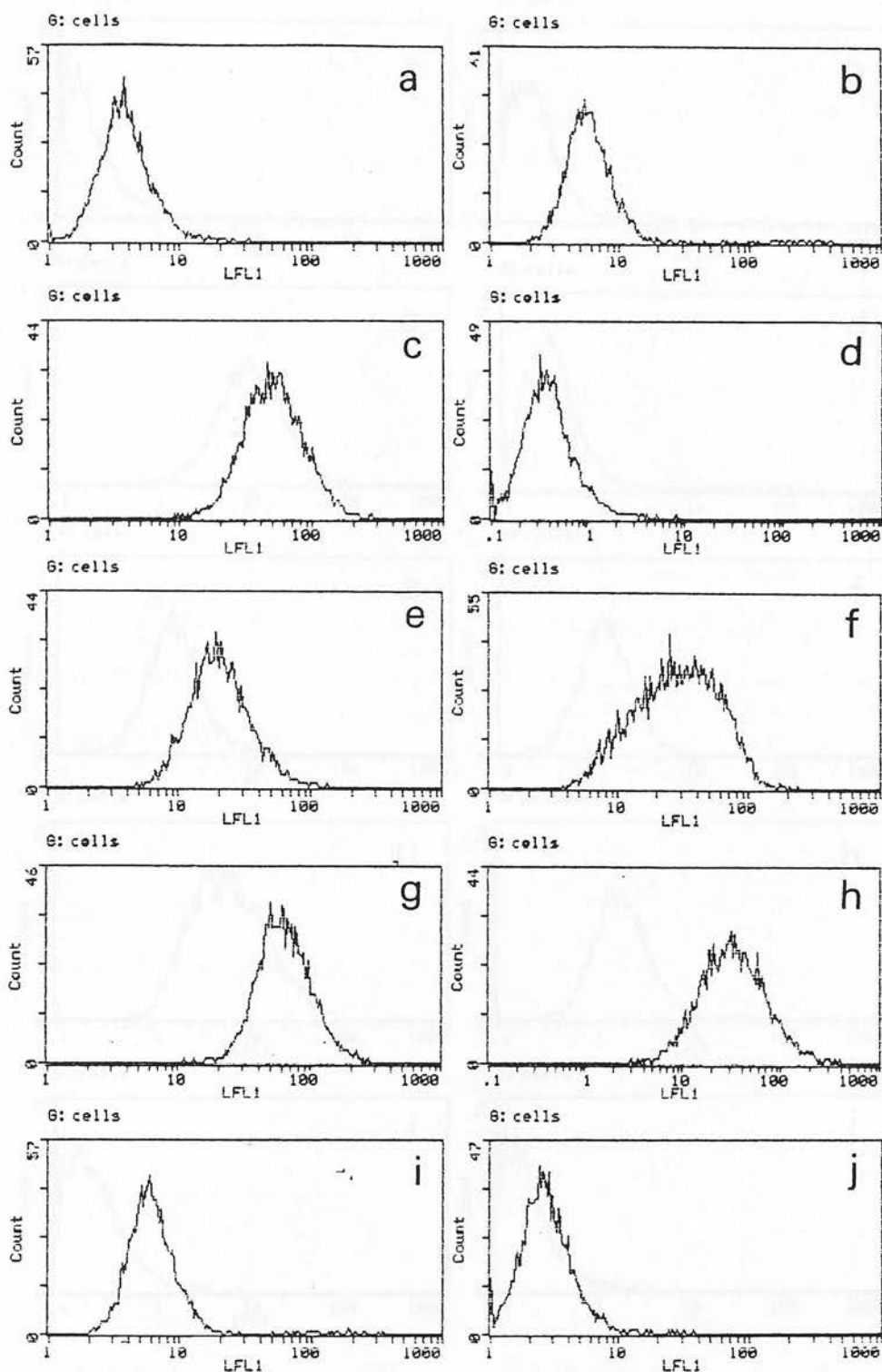


Figure 7.4. A typical example of the relative log fluorescence for each of the mAbs directed against the integrins a) mouse control, b) rat control, c) CDw49b, d) CDw49d, e) CDw49e, f) CDw49f, g) CD29, h) α_v , i) $\alpha_v\beta_3$, and j) 52 μ on the surface of the A549 epithelial cell.

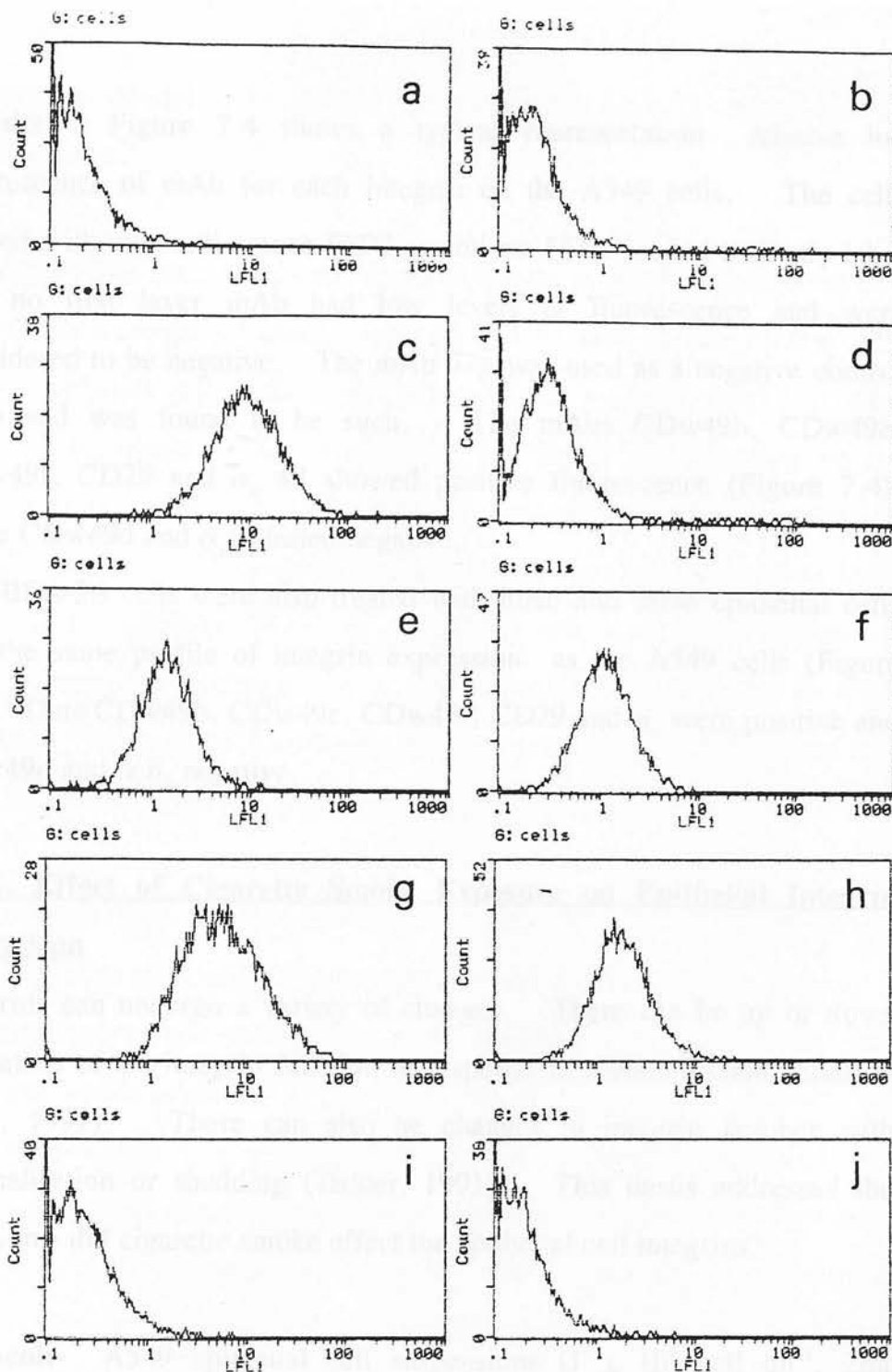


Figure 7.5. A typical example of the relative log fluorescence for each of the mAbs directed against the integrins a) mouse control, b) rat control, c) CDw49b, d) CDw49d, e) CDw49e, f) CDw49f, g) CD29, h) α_v , i) $\alpha_v\beta_3$, and j) 52 μ on the surface of the BEA-2B epithelial cell.

Results:- Figure 7.4 shows a typical representation relative log fluorescence of mAb for each integrin on the A549 cells. The cells treated with only anti-mouse FITC or anti-rat FITC second antibody label and no first layer mAb had low levels of fluorescence and were considered to be negative. The mAb 52 μ was used as a negative control mAb and was found to be such. The mAbs CDw49b, CDw49e, CDw49f, CD29 and α_v all showed positive fluorescence (Figure 7.4), while CDw49d and $\alpha_v\beta_3$ tested negative.

The BEA-2B cells were also treated with mAb and these epithelial cells had the same profile of integrin expression as the A549 cells (Figure 7.5). Thus CDw49b, CDw49e, CDw49f, CD29 and α_v were positive and CDw49d and $\alpha_v\beta_3$ negative.

7.3.1. Effect of Cigarette Smoke Exposure on Epithelial Integrin Expression

Integrins can undergo a variety of changes. There can be up or down regulation of the integrin function in response to certain stimuli (Spertini *et al*, 1991). There can also be changes in integrin number with internalisation or shedding (Tedder, 1991). This thesis addressed the question:- did cigarette smoke affect the epithelial cell integrins?

Protocol:- A549 epithelial cell suspensions (1×10^6 cell ml⁻¹, 2ml aliquots) were exposed to increased doses of both whole or vapour phase cigarette smoke and BEA-2B epithelial cell suspensions were exposed to the same concentrations of vapour phase cigarette smoke only (Section 2.2.4). Following exposure the cells were washed immediately in MEM (200g, 5 minutes), resuspended in wash buffer (2×10^5 cell ml⁻¹) and labelled with the mAb integrins to which the cells were positive using the

protocol as described in Section 2.2.8 with the negative control of 52μ also included.

Results:- The results are expressed as a ratio of the log fluorescence of the smoke-exposed or sham -exposed cell to that of the control cell. A549 epithelial cells which did not have an antibody label but were exposed to whole smoke alone have a relative high reading of log fluorescence (Table 7.1). This is a phenomenon known as autofluorescence and has been shown to occur on cells taken from lungs of cigarette smokers (Sköld *et al*, 1989), and may mask changes in fluorescence due to changes in integrin expression on exposure to whole smoke. There is an increase in the fluorescence of the four positive integrins when A549 epithelial cells were exposed to whole smoke (Table 7.1). However, the negative control of 52μ also showed a rise in fluorescence from baseline values giving probably a false positive result. The BEA-2B epithelial cells were not exposed to whole smoke because of the autofluorescence observed on A549 epithelial cells exposed to whole cigarette smoke alone.

Autofluorescence was not apparent when either type of epithelial cells were exposed to vapour phase cigarette smoke. There was no significant change in integrin expression ($p < 0.05$) with vapour smoke exposure of cells in comparison to the control cells of either epithelial cell lines A549 (Table 7.2) or BEA-2B (Table 7.3). It therefore appears that vapour phase cigarette smoke and probably whole cigarette smoke do not alter integrin expression. However, it is possible to alter integrin function without altering integrin expression.

| Integrin | Whole cigarette smoke (puffs) | | | |
|---------------|-------------------------------|-------------|-------------|--------------|
| | Sham | 1 | 2 | 4 |
| Control | 0.98 (0.02) | 6.15 (0.55) | 8.90 (2.46) | 16.40 (3.20) |
| Mouse Control | 0.97 (0.03) | 3.32 (0.55) | 4.82 (0.45) | 5.18 (1.04) |
| Rat Control | 1.01 (0.02) | 2.38 (0.42) | 2.91 (0.95) | 4.60 (0.43) |
| CDw49b | 1.09 (0.06) | 1.40 (0.28) | 1.34 (0.24) | 1.60 (0.39) |
| CDw49e | 1.06 (0.02) | 1.56 (0.17) | 1.81 (0.20) | 1.96 (0.17) |
| CDw49f | 0.99 (0.02) | 1.04 (0.06) | 1.12 (0.09) | 1.13 (0.09) |
| α_v | 0.96 (0.10) | 1.19 (0.02) | 1.20 (0.06) | 1.25 (0.08) |
| 52 μ | 1.04 (0.01) | 2.36 (0.82) | 4.38 (0.16) | 5.39 (0.46) |

Table 7.1. The effect of increasing exposure of A549 epithelial cells to whole cigarette smoke (1, 2, or 4 puffs) on integrin expression. The results are expressed as the ratio of the log fluorescence of the sham or smoke exposed cells to that of the unexposed cells. The data represents the mean (SEM) of three separate experiments.

| Integrin | Vapour phase cigarette smoke exposure (puff) | | | |
|---------------|--|-------------|-------------|-------------|
| | Sham | 1 | 2 | 4 |
| Control | 0.98 (0.02) | 1.14 (0.03) | 0.98 (0.02) | 1.02 (0.05) |
| Mouse Control | 0.97 (0.03) | 1.21 (0.06) | 1.16 (0.07) | 1.12 (0.05) |
| Rat Control | 1.01 (0.02) | 1.00 (0.17) | 1.09 (0.04) | 1.04 (0.02) |
| CDw49b | 1.09 (0.06) | 0.97 (0.05) | 1.01 (0.09) | 1.01 (0.10) |
| CDw49e | 1.06 (0.02) | 1.06 (0.02) | 1.16 (0.08) | 1.16 (0.13) |
| CDw49f | 0.99 (0.02) | 0.93 (0.04) | 1.01 (0.01) | 0.94 (0.03) |
| α_v | 0.96 (0.10) | 1.03 (0.06) | 1.04 (0.05) | 1.07 (0.04) |
| 52 μ | 1.04 (0.01) | 1.20 (0.06) | 1.26 (0.11) | 1.38 (0.11) |

Table 7.2. The effect of increasing exposure of A549 epithelial cells to vapour phase cigarette smoke (1, 2, or 4 puffs) on integrin expression. The results are expressed as the ratio of the log fluorescence of the sham or smoke exposed cells to that of the unexposed cells. The data represents the mean (SEM) of three separate experiments. There were no significant changes in integrin expression.

| Integrin | Vapour phase cigarette smoke exposure (puff) | | | |
|---------------|--|-------------|-------------|-------------|
| | Sham | 1 | 2 | 4 |
| Control | 1.07 (0.06) | 1.09 (0.07) | 1.09 (0.06) | 1.05 (0.03) |
| Mouse Control | 1.20 (0.09) | 1.08 (0.05) | 1.04 (0.02) | 1.01 (0.01) |
| Rat Control | 0.95 | 1.12 (0.03) | 1.02 (0.01) | 1.03 (0.01) |
| CDw49b | 1.11 (0.14) | 1.09 (0.07) | 1.16 (0.03) | 1.24 (0.10) |
| CDw49e | 1.08 (0.03) | 0.96 (0.01) | 0.93 (0.03) | 1.11 (0.02) |
| CDw49f | 1.18 (0.18) | 0.95 (0.04) | 0.97 (0.08) | 1.00 (0.02) |
| α_v | 1.01 (0.06) | 0.97 (0.05) | 0.97 (0.01) | 0.91 (0.05) |
| 52 μ | 1.11 (0.08) | 1.04 (0.04) | 1.08 (0.06) | 1.00 (0.05) |

Table 7.3. The effect of increasing exposure of BEA-2B epithelial cells to vapour phase cigarette smoke (1, 2, or 4 puffs) on integrin expression. The results are expressed as the ratio of the log fluorescence of the sham or smoke exposed cells to that of the unexposed cells. The data represents the mean (SEM) of four separate experiments. There were no significant changes in integrin expression.

7.3.2. Effect of Blocking of Integrins on Epithelial Attachment

It was necessary to establish which of the integrins were involved in promoting attachment of epithelial cells to culture plates. The following assay was carried out on the A549 cells only, because the BEA-2B cells are initially much slower at attaching.

Protocol:- The A549 epithelial cells were ^{51}Cr labelled for the attachment assay as described in Section 2.2.3a and resuspended at 4×10^6 cells ml^{-1} for blocking of the integrins. The cells were plated out into the flex-wells, spun at 250g for 1 minute and resuspended in MEM containing the relevant mAb at optimal dilution for flow cytometry or $\times 10$ optimal dilution. These were then left on ice (4°C) to allow saturation of the integrins with the mAb to occur. After 30 minutes the cells were diluted in medium containing the correct mAb to give a concentration of 5×10^5 cells ml^{-1} . Aliquots ($100\mu\text{l}$) of this suspension were then plated out in triplicate into a 96-well culture plate and incubated for 1 hour at 37°C , 5% CO_2 . The wells were gently washed twice with PBS and $200\mu\text{l}$ of triton-X-100 was added. The counts present in the triton-X-100 cell lysates were then measured on the γ -counter.

Results:- The first problem was a relative low percentage of A549 cell attachment at the 1 hour time point, and this is dealt with in greater detail in Chapter 3, Figure 3.1. The raw data was used to show any small changes that may have occurred. Extending the incubation time would result in internalisation of the integrin with attached mAb as integrins are active and are continually cycling. The new integrins would not be fully blocked and the cells could therefore attach more easily. The second problem was that cells treated with the negative control mAbs of

CDw49d and 52 μ showed signs of decreased cell attachment (Figure 7.6). This is non-specific blocking of attachment and will bias the lack of cell attachment affecting the interpretation of the results. Compared with control cells, optimum dilution mAb treated cells to α_v and β_1 integrins showed less cell attachment (Figure 7.6, panel a). It was therefore not possible to pursue this line of investigation because of the non-specific binding observed.

The A549 and BEA-2B epithelial cell lines do express certain integrins for ECM proteins and may well express others that have yet to be examined. The relevance of this data to epithelial cells *in vivo* needs to be addressed. Do the alveolar type II pneumocytes and bronchial epithelial cells in the lung express the same integrins? The following sections attempt to answer this question. Two approaches were taken. The first was to attempt to isolate human type II pneumocytes and characterise their integrins. The second was to assess the integrin profile in lung sections by immunohistochemistry.

7.4. Isolation of Human Type II Pneumocytes

Isolation of type II pneumocytes from freshly resected human lungs was attempted using the method of Bingle *et al*, (1990) which is described in Section 2.2.1d.

Results:- A number of problems were encountered. The first was the availability of tissue since there was a demand for the resected lungs by other research groups within the University. The second was the state of the tissue when acquired: the most suitable lungs were from patients with small peripheral tumours, however this further reduced availability. Some lungs had pneumonia distal to the tumour or had large and/or

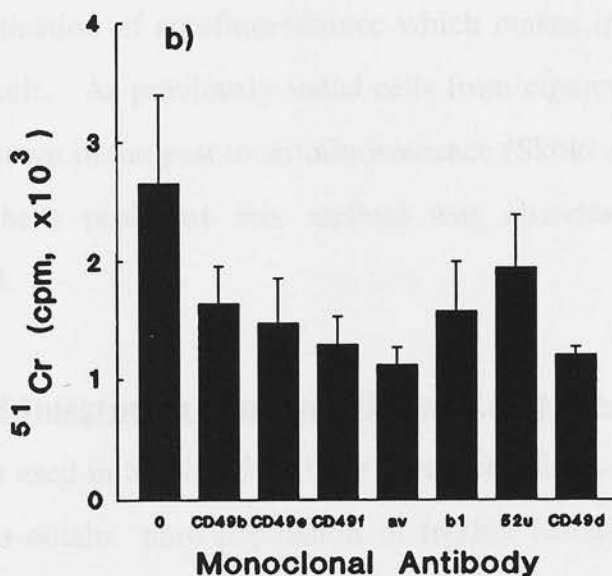
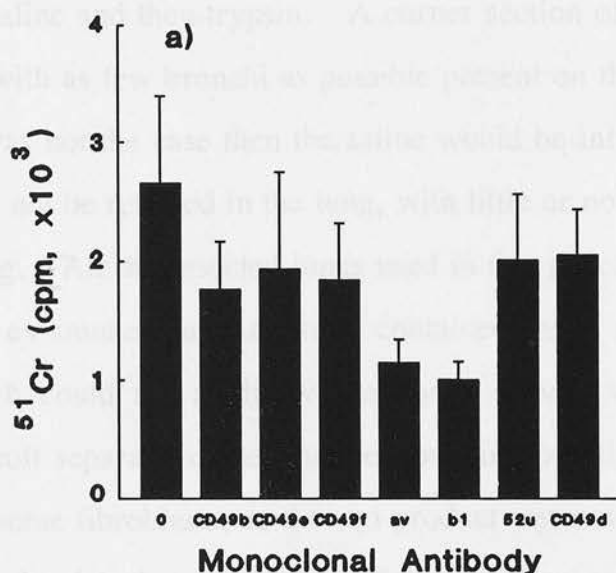


Figure 7.6. The effect of mAbs at a) optimal dilution, and b) $\times 10$ optimal dilution on A549 epithelial cell attachment to plastic, as measured in ^{51}Cr cpm. The histograms represent the mean of triplicate wells of three separate experiments and the bars indicate 1 SEM in each case.

necrotic tumours. If these first hurdles were successfully negotiated it was then necessary to have a piece of tissue that could easily be perfused and inflated with saline and then trypsin. A corner section of tissue was required for this, with as few bronchi as possible present on the dissected surface. If this was not the case then the saline would be infused in one bronchi and would not be retained in the lung, with little or no washing of the tissue occurring. All the resected lungs used in this procedure were from smokers or ex-smokers and as such contained large numbers of macrophages which could not all be washed out. Even with trypsin digestion and Percoll separation, the final cell preparation still contained macrophages and some fibroblasts, so the end product was not sufficiently pure to be used in the detachment assay. If these cell suspensions were used for mAb labelling and flow cytometry for integrin expression, there is the added complication of autofluorescence which makes interpretation of the results difficult. As previously stated cells from cigarette smokers' lungs have been shown in the past to autofluorescence (Sköld *et al*, 1989). As a result of these problems this method was abandoned and an alternative pursued.

7.5. Expression of Integrins in "Normal" Human Lung Tissue Sections

Although the mAb used in Section 7.3. have specificity for human cells it is very difficult to obtain pure population of freshly isolated type I or type II alveolar epithelial cells from human lungs. It is however possible to obtain human tissue sections and employ immunohistochemistry methods to stain for the cell integrins.

Protocol:- The method used was an ABCComplex -alkaline phosphatase stain with vector red substrate staining and the counterstain of

hematoxylin. The secondary antibodies were rabbit-anti-mouse biotin at 1:400 dilution and rabbit-anti-rat-biotin at 1:50 dilution. The staining was first attempted on paraffin sections of "normal" lung tissue and the same mAbs as those used for the flow cytometry. The mAbs used were those positive for flow cytometry against the integrin CDw49b, CDw49e, CDw49f, α_v and β_1 with the mAb to CDw49d which was negative on flow cytometry. The mAbs were used over a range of dilutions of 1:50 to 1:1000. A negative control with substitution of the first mAb with just buffer was also carried out.

Results for Paraffin Sections:- The "normal" lung tissue was actually scarred and undergoing hyperplasia. Very little staining was seen with the combination of mAbs and paraffin sections. The negative control showed no staining had occurred. Only CDw49e (serotec) was positively stained. When a high level of antibody was present (dilution of 1:100) there was general background staining with what appeared to be positive staining of type II pneumocytes (Figure 7.7, panel a). The lower dilution of 1:400 of the antibody reduced the background staining and the staining of the type II pneumocytes, although there was still positive staining of bronchial epithelium (Figure 7.7, panel b). A different set of mAbs (courtesy of Department of Pathology, University of Edinburgh) were then tried again on paraffin sections with little progress. This second set of antibodies paradoxically produced negative results on staining for the integrins CD29, CDw49e, CDw49f and α_v on the lung sections. Positive bronchial epithelial staining was observed with the antibody to CDw49b (Figure 7.8) and patchy staining was seen with CDw49d (Figure 7.9, panel a & b) with both a negative and positive area on the lung sections. This could purely be a problem with the method of fixation which was by

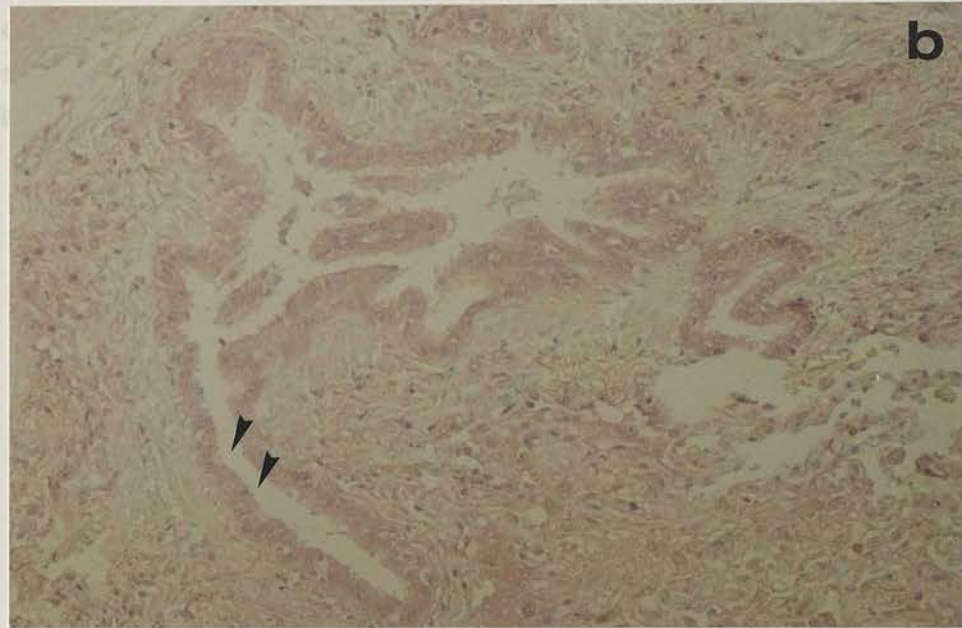
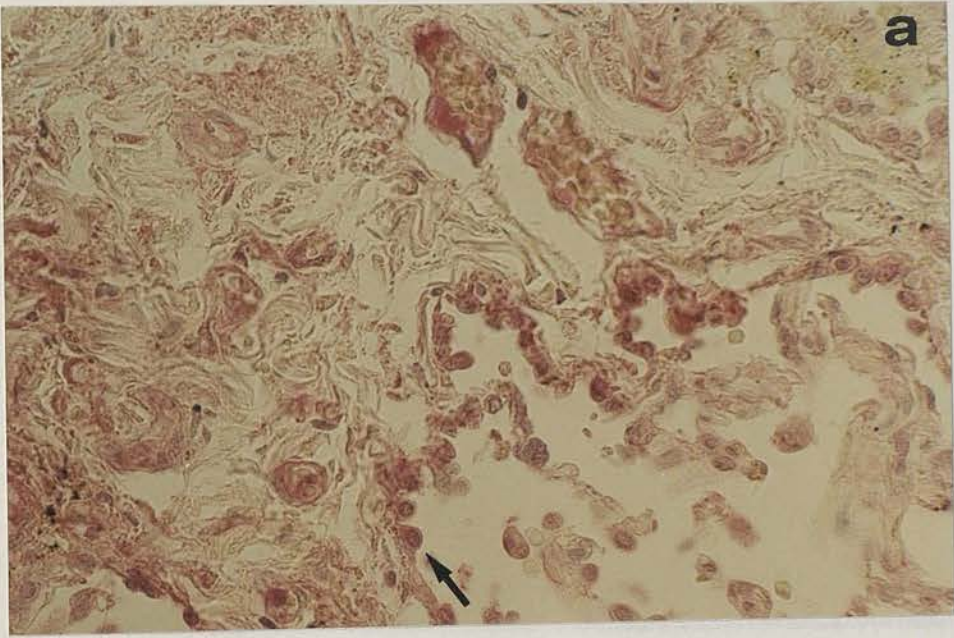


Figure 7.7. Light microscope image of a paraffin section of peripheral lung tissue immunostained with mAb directed against CDw49e at a dilution of a) 1:100 and b) 1:400. The type II pneumocytes (arrows) and the bronchial epithelium (arrowhead) expressed CDw49e. (Magnification a) x250; b) x100)

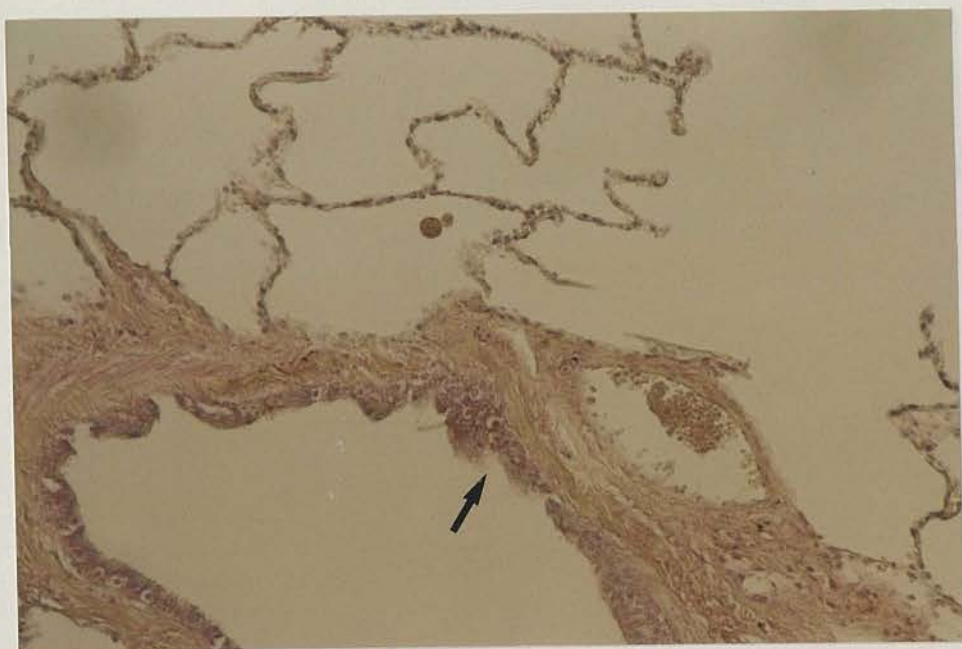


Figure 7.8. Light microscope image of a paraffin section of peripheral lung tissue immunostained with mAb directed against CDw49b. There was positive staining of the bronchial epithelium (arrow). (Magnification x100)

Figure 7.8. Light microscope image of a paraffin section of peripheral lung tissue immunostained with mAb directed against CDw49b. There was positive staining of the bronchial epithelium (arrow). (Magnification of x250; b) x100)

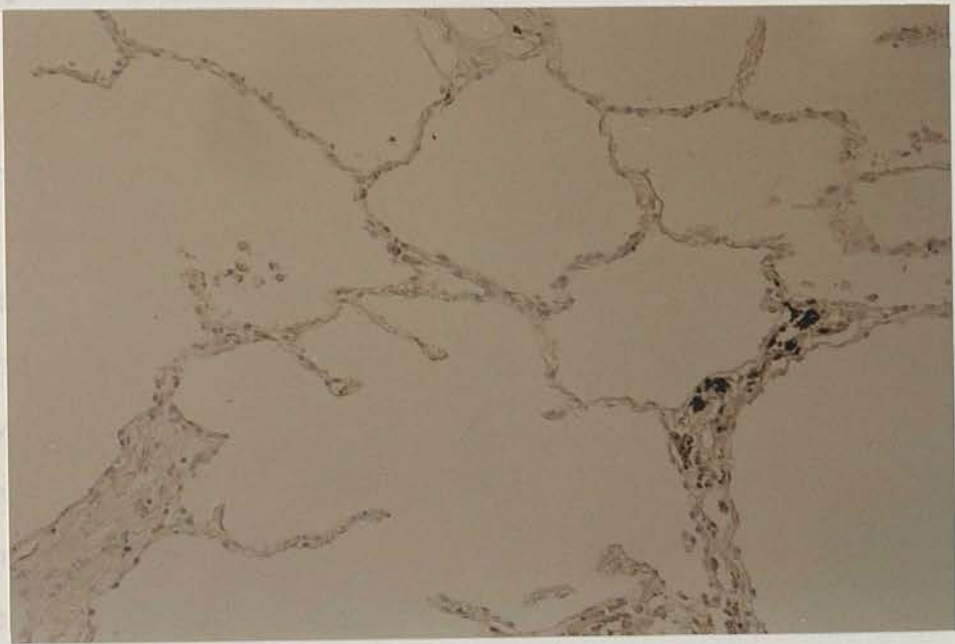
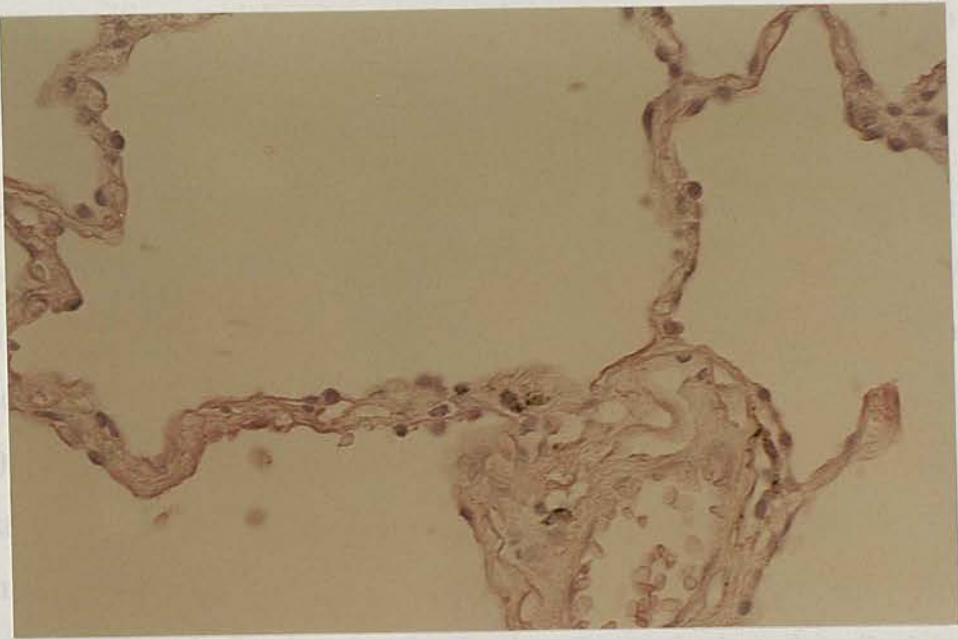


Figure 7.9. Light microscope image of a paraffin section of peripheral lung tissue immunostained with mAb directed against CDw49d. There were areas of a) positive staining at the alveolar epithelial cell level and b) negative staining (Magnification a) x250; b) x100)

using formaldehyde. The next step was to try a different type of section, i.e. frozen section, which may preserve the epitope lost on processing to paraffin.

Protocol for Preparation of Frozen Section:- These experiments involved collecting a fresh lung specimen from thoracic surgical theatre, the patients undergoing lung resection for bronchial carcinoma. The pathologist then dissected out pieces of "normal" lung tissue (5-10mm cubed) distant from the tumour and these were embedded in OCT (this is a mixture of polyvinyl alcohol, polyethylene glycol, dimethyl benzyl ammonium chloride), and frozen quickly (-70°C, overnight). These were then sectioned, fixed and stained using the method as described in the section for the paraffin sections.

Results for Frozen Sections:- The staining of the frozen section produced conflicting evidence on integrin expression in human lung. There was no staining observed on the negative control (Figure 7.10, panel a). The staining was still negative for the integrins for α_v , with positivity for CD29, CDw49b, CDw49d, CDw49e and CDw49f. However, there was a general heavy non-specific red wash on the lung section incubated with the CD29 mAb (Figure 7.10, panel b). A lung section was membrane positive on bronchial epithelial cells for CDw49b but negative for alveolar epithelium (Figure 7.11, panel a). This epithelium was also negative for CDw49d but the interstitial macrophages stained positively (Figure 7.11, panel b). With mAb to CDw49e again a general non-specific staining was observed with vessel but no alveolar-specific staining present (Figure 7.12, panel a). There was weak non-specific staining for CDw49f (Figure 7.12, panel b). Examination of

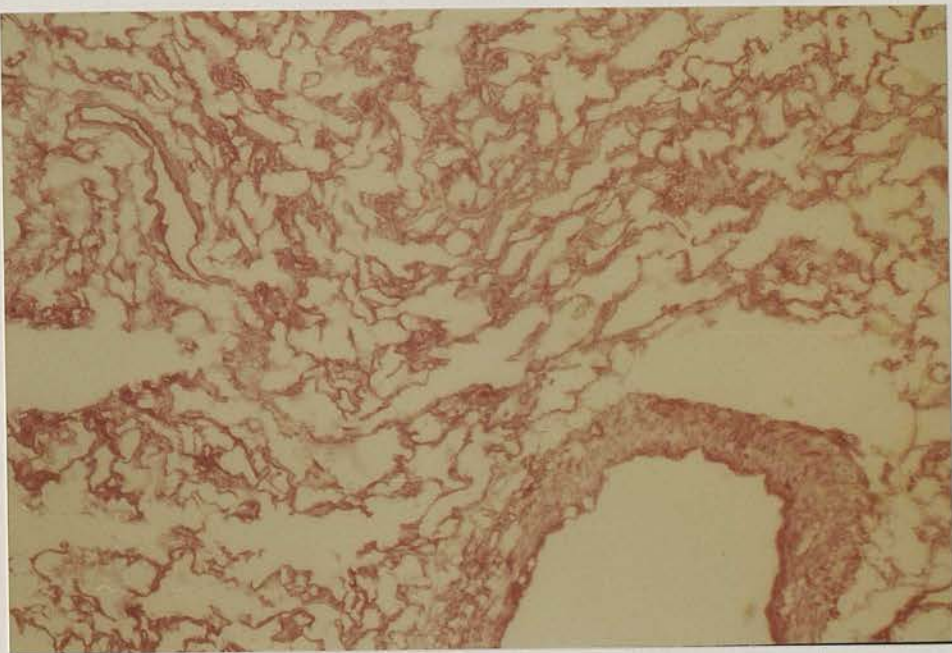


Figure 7.10. Light microscope image of a frozen section of peripheral lung tissue immunostained a) negative control b) mAb directed against CD29. There was no reactivity in the negative control and a general non-specific staining for CD29. (Magnification a) x100; b) x40)

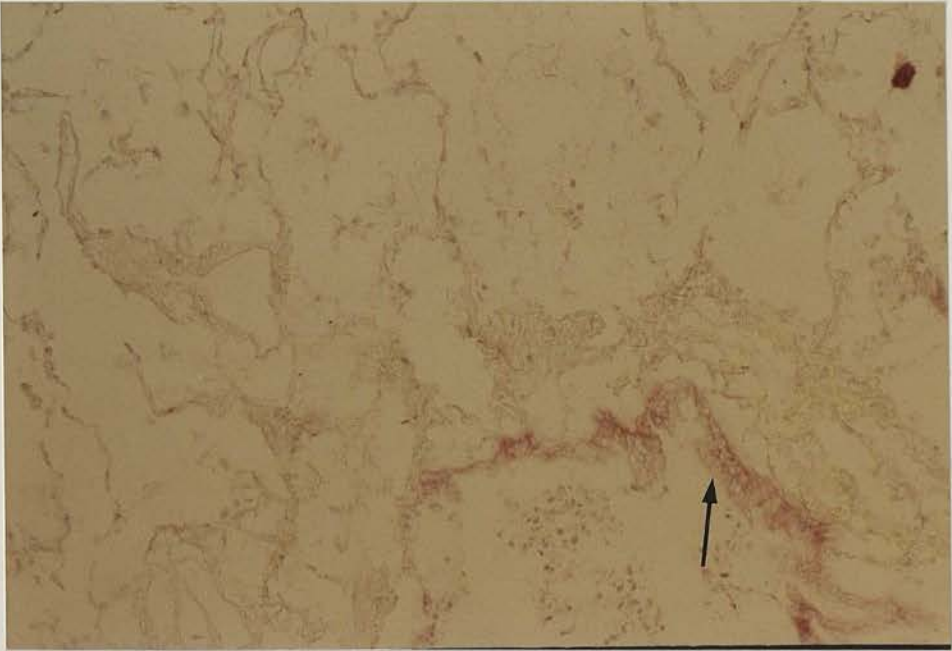


Figure 7.11. Light microscope image of a frozen section of peripheral lung tissue immunostained with mAbs directed against a)CDw49b and b) CDw49d. The bronchial epithelium (arrows) expressed CDw49b with interstitial macrophages (arrowheads) expressing CDw49d. (Magnification a) x100;

b) x250)

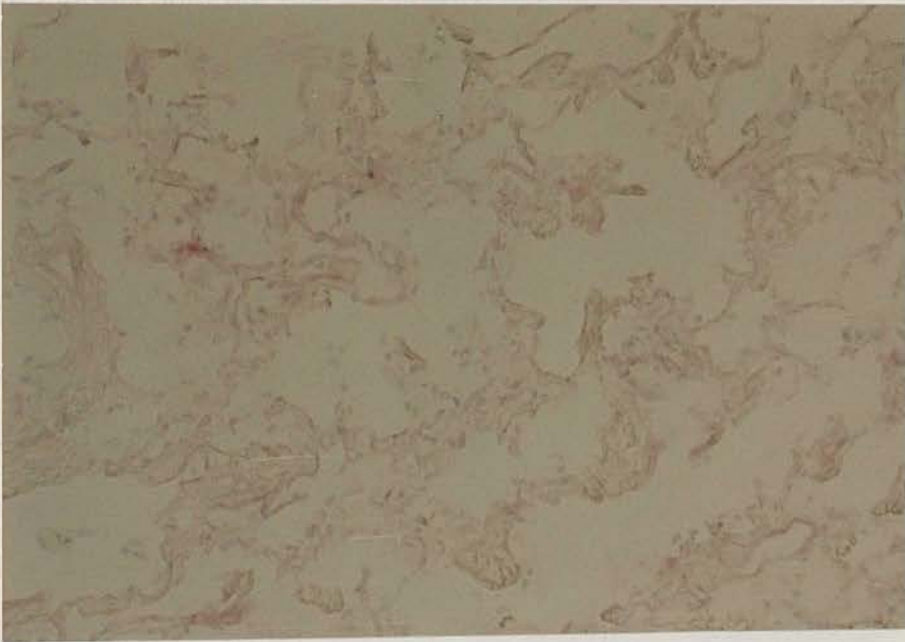
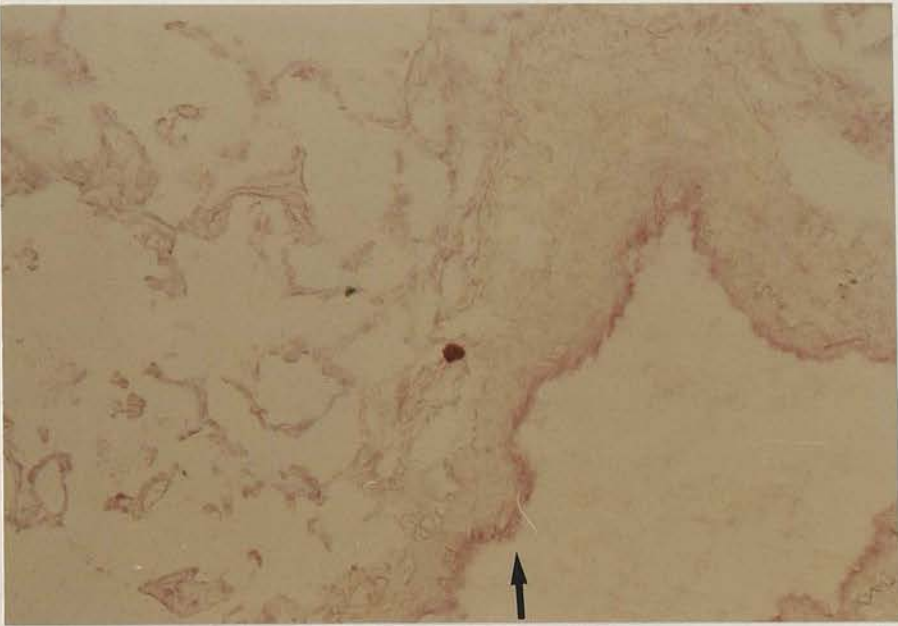


Figure 7.12. Light microscope image of a frozen section of peripheral lung tissue immunostained with mAbs directed against a) CDw49e and b) CDw49f. There was weak general staining for CDw49e with stronger positivity of the vessel endothelium (arrows). There was also weak non-specific staining for CDw49f. (Magnification a) x100; b) x100)

these tissue sections was cross checked by the lung pathologist, Dr. W. Wallace, Dept. of Pathology, University of Edinburgh, who confirmed the findings.

SUMMARY

The attachment of A549 and BEA-2B epithelial cells was cation-dependent and there was a trend towards increased A549 epithelial cell detachment on incubation with RGD. Both types of cells were positive for the integrins CDw49b, CDw49e, CDw49f, α_v and β_1 , but negative for CDw49d and $\alpha_v\beta_3$. Using a mAb blocking assay, there was found to be possible involvement of both α_v and β_1 in the attachment of A549 cells. Upon exposure to cigarette smoke there was no change in integrin expression, but this does not rule out change in function.

Care must be taken in drawing conclusions from the staining patterns of the mAbs on the lung sections examined. Paraffin sections showed very little staining while the frozen sections were better for staining. General non-specific staining, which was observed, may be masking more specific staining or giving a false positive to certain cell types. The negative results observed may well be false negatives for a variety of reasons and should also be interpreted with caution.

Attachment

The microtubular component of the cytoskeleton can be disrupted by a variety of agents, such as colchicine and vinorelbine. Colchicine was used in this study to disrupt the relationship between the microtubules and epithelial detachment. It is an alkaloid extracted from the meadow saffron and has been used as a treatment for gout. It disrupts the microtubules by binding the protofilaments of tubulin to one another slower and thereby preventing its polymerisation.

CHAPTER 8

CYTOSKELETON.

In Chapter 4 the A549 epithelial cells were observed to change their shape by rounding up in response to CSC. The A549 epithelial cells are eucaryotic cells and as such have a distinct shape and a high degree of internal organisation. These cells are capable of changing their shape, redistributing their intracellular organelles and moving from one position to another, which are all necessary for the process of repair. These events are dependent on a complex network of protein filaments in the cytoplasm which are known collectively as the cytoskeleton. The work in this chapter assesses the role of the cytoskeleton in CSC induced A549 epithelial cell detachment, with the emphasis on two of the most important components, the microtubules and the microfilaments.

8.1. MICROTUBULES

8.1.1. Effect of Microtubular Disruption on A549 Epithelial Cell Detachment

The microtubular component of the cytoskeleton can be disrupted by a variety of agents, such as colchicine and vinblastine. Colchicine was used in this study to examine the relationship between the microtubules and epithelial detachment. It is an alkaloid extracted from meadow saffron and has been used as a treatment for gout. It disrupts the microtubules by binding one molecule of colchicine to one tubulin dimer and thereby preventing its polymerisation.

Protocol:- The A549 epithelial cells were prepared for the detachment assay. The following morning the cells were washed twice with PBS (200 μ l aliquots) and colchicine added in triplicate wells in increasing concentrations (10,25,50,100, 200 and 500 μ M). These cells were then incubated at 37°C, 5%CO₂ for 4 hours.

Results:- Figure 8.1, panel a, shows that colchicine, at concentrations of 10-500 μ M, had no effect on A549 epithelial detachment or cell lysis.

8.2. MICROFILAMENTS

8.2.1. Effect of Microfilament Disruption on Epithelial Detachment

The family of alkaloids known as the cytochalasins have been used by a number of groups to study the role of the microfilament structure in the cell. The cytochalasins were found to bind specifically to actin leading to fragmentation of the micro-filaments. There is a wide range of affinity for actin for the different members of the cytochalasins family (Lin & Lin, 1979).

Protocol:- The A549 cells in the epithelial detachment assay were treated with increasing concentrations of two of the compounds in this family, cytochalasin B and cytochalasin D (0.1, 0.2, 0.5, 1, 2 and 5 μ M) during a standard incubation time of 4 hours at 37°C, 5% CO₂.

Results:- After 4 hours incubation there was an increase in epithelial cell detachment with both treatments. The A549 cells were more sensitive to cytochalasin D with significant changes in detachment levels beginning at concentrations of 1 μ M (Figure 8.1, panel c) whereas significant

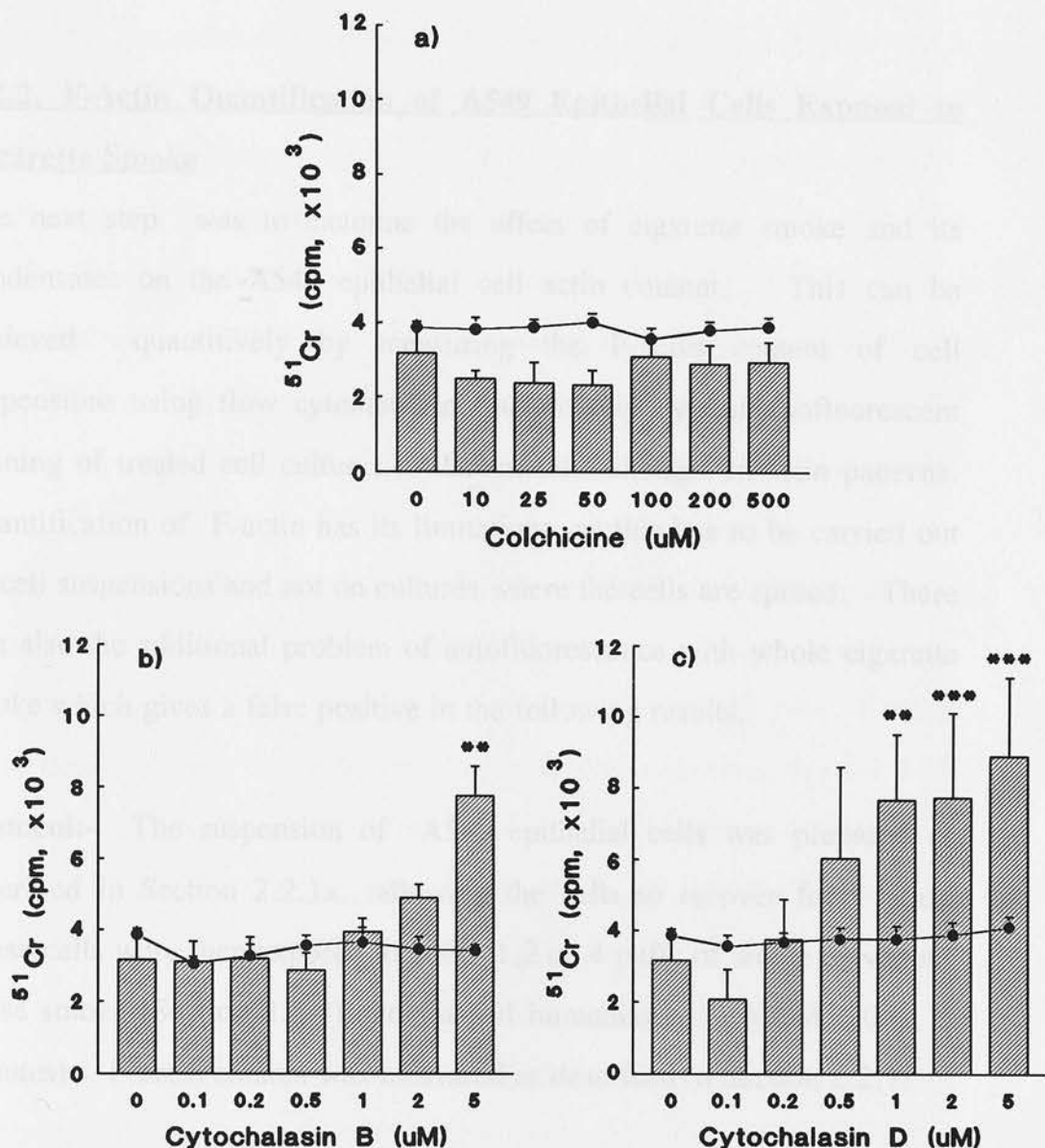


Figure 8.1. The effect of increasing concentrations of a) Colchicine, b) Cytochalasin B, and c) Cytochalasin D on the detachment (histograms) and lysis (●) of A549 epithelial cells, measured in ^{51}Cr cpm. The histograms/● represent the mean of triplicate wells in four separate experiments and the bars 1 SEM in each case. ** $p < 0.01$; *** $p < 0.001$ compared with medium alone (0).

detachment did not occur until concentrations of $5\mu\text{M}$ with cytochalasin B (Figure 8.1, panel b).

8.2.2. F-Actin Quantification of A549 Epithelial Cells Exposed to Cigarette Smoke

The next step was to examine the effect of cigarette smoke and its condensates on the A549 epithelial cell actin content. This can be achieved quantitatively by measuring the F-actin content of cell suspensions using flow cytometry or quantitatively by immunofluorescent staining of treated cell cultures to demonstrate changes in actin patterns. Quantification of F-actin has its limitations, as this has to be carried out on cell suspensions and not on cultures where the cells are spread. There was also the additional problem of autofluorescence with whole cigarette smoke which gives a false positive in the following results.

Protocol:- The suspension of A549 epithelial cells was prepared as described in Section 2.2.1a., allowing the cells to recover for 1 hour. These cells were then exposed to either 1,2 or 4 puffs of whole or vapour phase smoke (Section 2.2.4) and washed immediately in MEM (200g, 5 minutes). F-actin content was measured as described in Section 2.2.7.

Results:- The A549 epithelial cells that were either untreated or sham exposed gave approximately the same mean log fluorescence for each experiment with a range of 3-32. The mean log fluorescence of the whole smoke exposure experiment for untreated control cells was 9.6 ± 5.5 , and for sham exposed cells 16.5 ± 3.6 ; whilst in the experiment using vapour smoke these control values were 12.2 ± 2 for control cells and 17.2 ± 4.4 for sham exposed cells. Upon exposure to whole smoke

there was an increase in F-actin content with 1, 2 and 4 puffs of smoke (Figure 8.2, panel a). This increase in F-actin seemed to be dose-dependent, the mean for 1 puff of whole smoke being 46.9 ± 5.6 with a range of 30-59, for 2 puffs the values were: mean 54.9 ± 10.2 and range 39-99, and for 4 puffs the mean was 71.6 ± 15 , with a range of 40-133. When exposed to vapour phase there was an increase in F-actin content in A549 cells but this was not dose-dependent or as large a change as compared with whole smoke. In this case the values were vapour 1 puff: mean 27.6 ± 5.7 , range 12-46; 2 puffs: mean 25.6 ± 6.8 , and range 12-54; and 4 puffs: mean 27.5 ± 6.5 , and range 16-55 (Figure 8.2, panel b).

8.2.3. Effect of Post Incubation of Cytochalasin on Cigarette Smoke Exposed A549 Epithelial Cells F-Actin Content

The cytochalasin was added in theory to disrupt the F-actin. Cytochalasin D was the preferred compound to disrupt actin as it has been proven in the past to have a higher affinity for F-actin (Lin & Lin, 1979) and was found to have a more potent effect on A549 epithelial cell detachment than cytochalasin B (Section 8.2.1.).

Protocol:- In the following experiment the A549 epithelial cells were harvested, allowed to recover and then exposed to either whole or vapour phase cigarette smoke in increasing doses (1, 2 or 4 puffs), immediately after which these cells were washed with MEM (200g, 5 minutes), cytochalasin D ($5\mu\text{M}$) was added and the cells were incubated for 30 minutes at 37°C . The F-actin content was measured as described in Section 2.2.7a.

Results:- Upon exposure to both types of cigarette smoke there was an

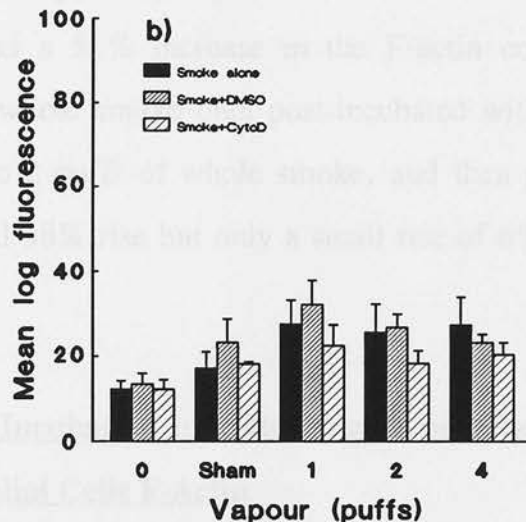
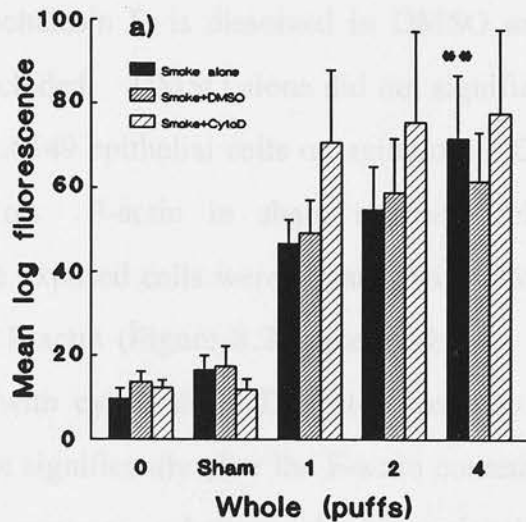


Figure 8.2. The effect of pre-incubation of cytochalasin D on the F-actin content of A549 epithelial cells exposed to increasing concentrations of a) whole, and b) vapour phase cigarette smoke, measured in log fluorescence. The histograms represent the mean of four separate experiments and the bars indicate 1 SEM in each case. ** $p < 0.01$ compared with sham exposure.

increase in F-actin, although this increase was greater with the whole rather than the vapour phase cigarette smoke, as described in the results Section 8.2.3. Cytochalasin D is dissolved in DMSO and therefore a DMSO control was included. DMSO alone did not significantly alter the F-actin content of the A549 epithelial cells on agitation. Cytochalasin D also had no effect on F-actin in sham exposed cells ($p > 0.05$). Furthermore, if smoke exposed cells were treated with DMSO there was no change in level of F-actin (Figure 8.2, panel a & b). Incubation of A549 epithelial cells with cytochalasin D post-exposed to vapour phase cigarette smoke did not significantly alter the F-actin content (Figure 8.2, panel b), although by contrast whole smoke-exposed cells had greater levels of fluorescence, suggesting higher levels of F-actin (Figure 8.2, panel a). There was a 51% increase in the F-actin content of cells exposed to 1 puff of whole smoke then post-incubated with cytochalasin D. When exposed to 2 puffs of whole smoke, and then post-incubated there was an additional 38% rise but only a small rise of 6% with 4 puffs of whole smoke.

8.2.5. Effect of Agitation CSC on F-Actin Content of A549 Epithelial

8.2.4. Effect of Pre-Incubation of Cytochalasin on Cigarette Smoke Exposed A549 Epithelial Cells F-Actin

In these studies A549 epithelial cells were pre-incubated with cytochalasin D to disrupt F-actin and to assess whether this binding of cytochalasin D with actin then prevented a rise in the F-actin content in the cells exposure.

Protocol:- The highest dose of 4 puffs of cigarette smoke was chosen for this experiment in which A549 epithelial cells were harvested and pre-incubated with 5 μ M cytochalasin D for 30 minutes at 37° C, 5% CO₂.

Thereafter the cells were exposed to either 4 puffs of whole or vapour phase smoke, washed in MEM (200g, 5 minutes) and the F-actin content of the cells was measured.

Results:- The F-actin content of cell suspensions untreated or sham exposed was higher than the previous experiment, as was the F-actin content of whole smoke-exposed cells. The control value for untreated cells in the whole cigarette smoke exposure experiment was 20.9 ± 5 and this rose to 111.7 ± 15.2 on exposure to 4 puffs of whole cigarette smoke. Sham exposure did not change the F-actin content (19.7 ± 3.9 $p > 0.05$). Cells that had been pre-incubated with cytochalasin D, which were then exposed to 4 puffs of whole cigarette smoke, had a lesser rise in F-actin compared with smoke exposed cells with no pre-incubation with cytochalasin D (84.3 ± 11) (Figure 8.3, panel a). By contrast there was no significant change in F-actin content in the cells exposed to vapour phase cigarette smoke. ($p > 0.05$), (Figure 8.3, panel b).

8.2.5. Effect of Aqueous CSC on F-Actin Content of A549 Epithelial Cells

Protocol:- A549 epithelial cells were harvested and allowed to recover as described in Section 2.2.1a. The cell suspensions were then treated with either whole or vapour CSC in increasing concentrations (0.1, 1, 5, or 10%) for 30 minutes. This time point was chosen because of the results of cell detachment which was observed within only 30 minutes following incubation with CSC. Although these cells are treated in teflon wells to slow down reattachment, this does still occur in the teflon wells with time. Up to 4 hours treatment was tried but the number of cells recovered from the teflon wells was minimal. After 30 minutes the cells

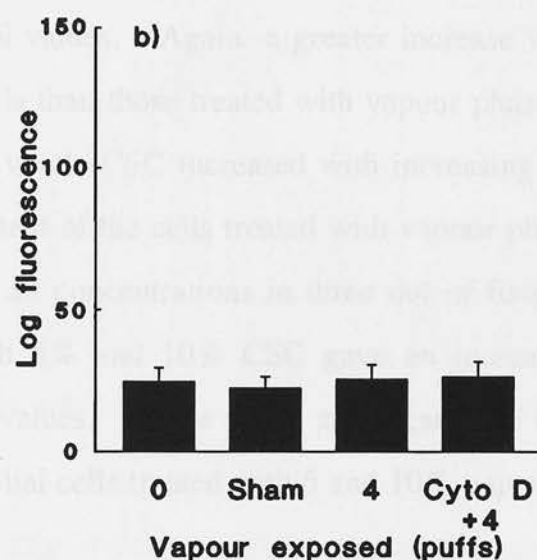
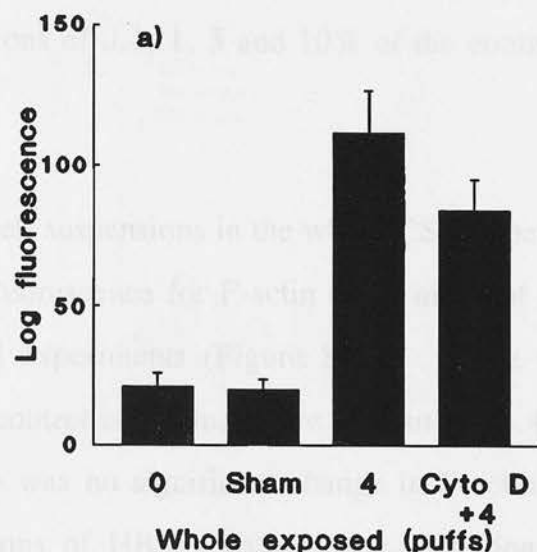


Figure 8.3. The effect of 30 minutes pre-incubation with cytochalasin D (5uM) on the F-actin content of A549 epithelial cells exposed to a) whole, and b) vapour phase cigarette smoke. The histograms represent the mean of four separate experiments and the bars indicate 1 SEM.

were washed in MEM (200g, 5 minutes) and the F-actin content measured as described in the second part of Section 2.2.7. The cells were also exposed to concentrations of 0.1, 1, 5 and 10% of the control solution of HBSS.

Results:- Untreated cell suspensions in the whole CSC experiment gave a similar range of log fluorescence for F-actin (2-51 mean of 20 ± 8.2) as in the smoke exposed experiments (Figure 8.4). In the vapour phase CSC experiments the control cell values were a mean of 11.4 ± 2.6 and a range of 2-17. There was no significant change in F-actin content with increasing concentrations of HBSS ($p > 0.05$). The log fluorescence value for all concentrations of whole and vapour phase CSC were higher than that of the control values. Again a greater increase was seen with whole CSC treated cells than those treated with vapour phase CSC. The F-actin content of the whole CSC increased with increasing dose (Figure 8.4). The F-actin content of the cells treated with vapour phase CSC was higher than control at all concentrations in three out of four experiments and in the fourth both 5% and 10% CSC gave an increase in F-actin content above control values. There was a significant rise in the F-actin content of A549 epithelial cells treated with 5 and 10% vapour phase CSC (Figure 8.4).

8.2.6. Effect of Cytochalasin and Aqueous CSC on F-Actin Content of A549 Epithelial Cells

Protocol:- Preliminary experiments where A549 epithelial cell were pre-incubated with cytochalasin D and then exposed to cigarette smoke produced conflicting results on the F-actin content of the cells. Thus in the experiments cigarette smoke condensates were combined with the

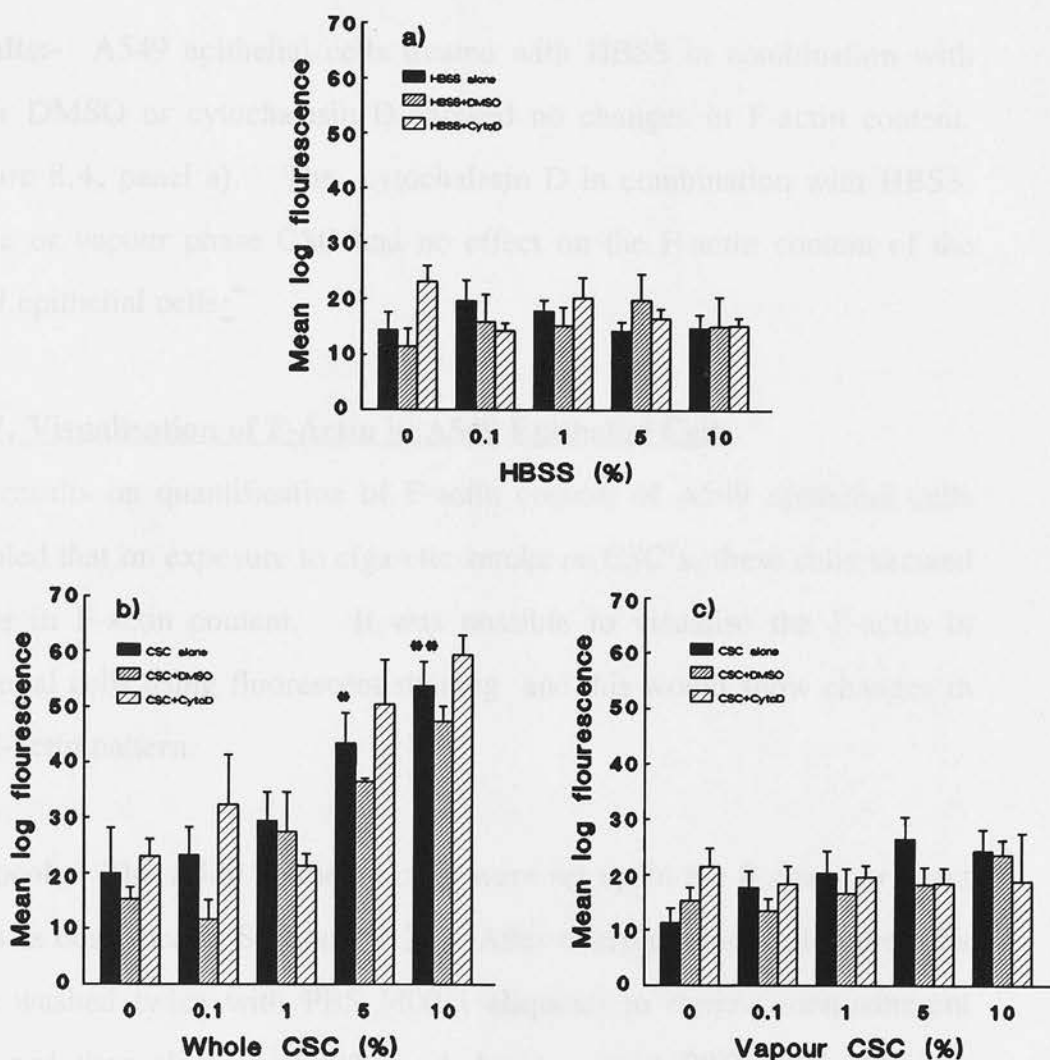


Figure 8.4. The effect co-incubation of cytochalasin D with increasing concentrations of a) HBSS, b) whole CSC, and c) vapour CSC on the F-actin content of A549 epithelial cells, measured in log fluorescence. The histograms/ represent the mean of four separate experiments and the bars indicate 1 SEM in each case. * $p < 0.05$; ** $p < 0.01$ compared with medium alone (0).

cytochalasin D 5 μ M and co-incubated for 30 minutes.

Results:- A549 epithelial cells treated with HBSS in combination with either DMSO or cytochalasin D showed no changes in F-actin content. (Figure 8.4, panel a). The cytochalasin D in combination with HBSS, whole or vapour phase CSC had no effect on the F-actin content of the A549 epithelial cells.

8.2.7. Visualisation of F-Actin in A549 Epithelial Cells

The results on quantification of F-actin content of A549 epithelial cells revealed that on exposure to cigarette smoke or CSC's, these cells showed a rise in F-actin content. It was possible to visualise the F-actin in epithelial cells using fluorescent staining and this would show changes in the F-actin pattern.

Protocol:- The A549 epithelial cells were set up in the 8 chamber glass slides as described in Section 2.2.3e. After overnight incubation the cells were washed twice with PBS (400 μ l aliquots) to remove non-adherent cells and then aliquots of either whole or vapour CSC (10% dilution, 400 μ l) were added to the cells. The control solutions of MEM or HBSS (10%) in MEM were also added to the chamber slides. These were then incubated for 2 hours at 37°C, 5% CO₂, the supernatants removed, the cells washed twice with PBS (400 μ l aliquots) again to remove non-adherent cells and the cells fixed in acetone for 10 minutes at room temperature. These treated cells were stained for F-actin with rhodamine-conjugated phalloidin as described in Section 2.2.7b.

Results:- A549 epithelial cells incubated in either MEM or MEM with

10% HBSS had similar staining patterns. These cells were well spread with, the staining was bright and there was a predominance of perinuclear staining (Figure 8.5, panel a for MEM; Figure 8.5, panel b for HBSS). When treated with either whole or vapour CSC the F-actin staining pattern was different from that of control cells. There was loss of the perinuclear staining and with both the whole and vapour CSC treated cells there was increased peripheral edge actin staining. The actin pattern was diffuse in the whole CSC treated cells (Figure 8.5, panel c). There were stress fibres prominent in the cells treated with vapour CSC (Figure 8.5, panel d). Thus exposure to cigarette smoke in the form of condensates led to a change in the actin patterns of adherent A549 epithelial cells when compared to unexposed A549 epithelial cells.

SUMMARY

Disruption of microtubules using colchicine had no effect on A549 epithelial cell detachment whereas disruption of the microfilaments led to a dose-dependent increase in A549 epithelial cell detachment. There was a dose-dependent increase in the F-actin content of A549 epithelial cells exposed to either whole cigarette smoke or whole CSC, although as previously mentioned this was probably an artificial result due to autofluorescence. Exposure to monolayers of A549 epithelial cells to either whole or vapour CSC led to a change in the actin staining patterns for cells, from the predominant perinuclear staining of control cells to the peripheral actin bands in CSC exposed cells.

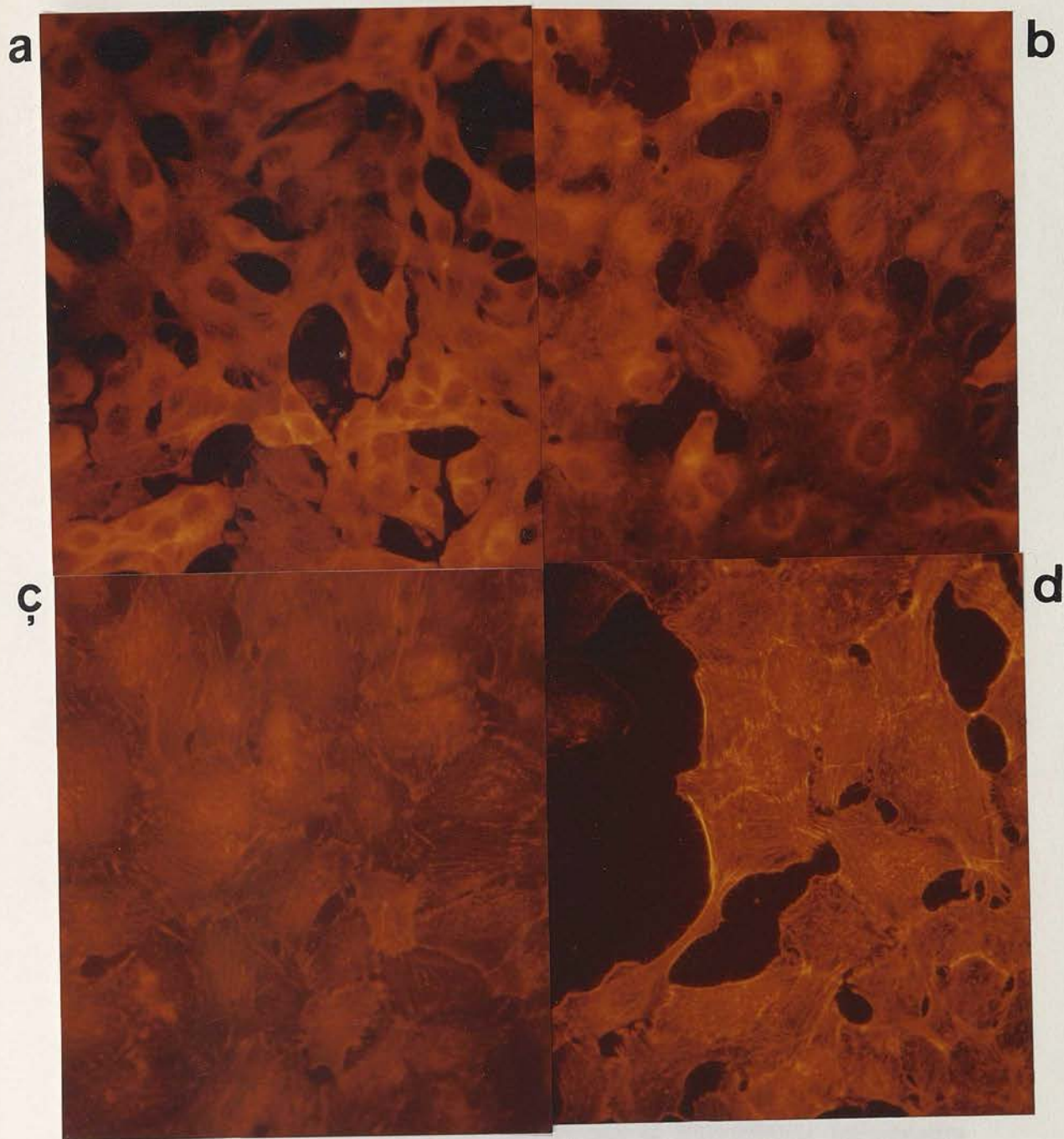


Figure 8.5. Fluorescent microscope image of A549 epithelial cells treated with a) MEM, b) 10% HBSS, c) 10% whole CSC, d) 10% vapour CSC for 2 hours, and then stained with Rhodamine-conjugated Phalloidin to demonstrate actin structure. Both MEM and HBSS treated cells (panels a and b) had perinuclear staining actin. The CSC treated cells had increased actin at the peripheral edges (panels c and d) with prominent stress fibres evident in vapour CSC treated cells (panel d). (Magnification x40).

CHAPTER 9

SIGNAL TRANSDUCTION

All cells are exposed to a myriad of different types of external signals from hormones, neurotransmitters, metabolites, extracellular structures such as the extracellular matrix, to natural and foreign chemicals. Pathways of intracellular communication are required to co-ordinate the basic functions of each cell and to provide links between these extracellular signals and intracellular responses:- the so called signal transduction pathways.

Key observations which laid the foundation for an understanding of the processes of intracellular communication were the discovery of cyclic AMP, expansion of the role of calcium (Ca^{2+}), the discovery of the protein kinases, which are discussed in Chapter 1, and elucidation of the function of the sequence-specific DNA-binding receptors. There are three main mechanisms by which external signals are received by cells. These are (1) receptors located on the external surface of the plasma membrane, (2) sequence-specific DNA-binding receptor proteins, and (3) certain enzymes in pathways of intermediary metabolism. These external signals once received can mobilise a series of intracellular messengers which transfer information to intracellular target enzymes. The following section of this thesis focuses on the role of protein kinases and intracellular Calcium ($[\text{Ca}^{2+}]_i$) in epithelial detachment and the effect of inhibition of protein kinases in response to CSC.

9.1. ROLE OF PROTEIN KINASES IN A549 EPITHELIAL CELL DETACHMENT

It is possible to either activate or inhibit the protein kinases by a variety of different compounds. All the following work was carried out using the aqueous CSC in the detachment assay with A549 epithelial cells.

9.1.1. Effect of Activation of cAMP-Dependent Protein Kinase on Aqueous CSC Induced A549 Epithelial Cell Detachment

Cell signalling can be altered by increasing intracellular cAMP levels. This serves as an intracellular signal (Sutherland, 1972) and activates cAMP-dependent protein kinase. This increase can be achieved experimentally using the compound Di butyryl cAMP (Bt₂cAMP), which is a congener of cAMP containing two butyryl groups attached to cAMP which facilitates cell entry through the plasma membrane. Once inside the cell the butyryl groups are cleaved and the molecule functions essentially as cAMP (Falbriard *et al*, 1967; Henion *et al*, 1967). This compound has been shown to raise the intracellular level of cAMP in vitro (Goldberg *et al*, 1974) and has been shown to block endotoxin induced lung injury in rats (Chang *et al*, 1989).

Another way to activate these kinases is to use Forskolin, which directly activates adenylate cyclase (Seamon *et al*, 1981), resulting in an increase in intracellular cAMP. Forskolin was used in concentrations of 25µM, which other research groups (Lew *et al*, 1992) found to be biologically active and non-toxic to cells.

Protocol of Activation by cAMP:- The A549 epithelial cells were prepared for the detachment assay and incubated overnight. The

following morning the cells were washed twice with PBS (200 μ l aliquots) and Bt₂ cAMP was added to each well in increasing concentrations (100 μ l aliquots, 10⁻⁶-10⁻³ M). The cells were incubated for 30 minutes and then the CSC's were added in to give a final concentration of 10% CSC. This was then incubated for 4 hours at 37°C, 5% CO₂ and the cells were harvested.

Results:- The addition of Bt₂ cAMP alone to A549 epithelial cells at concentrations of 10⁻⁶-10⁻³M did not produce any significant changes in background cell detachment or lysis (Figure 9.1, panel a). This was also the case for cells incubated with cAMP and 10% HBSS, which is the aqueous solvent used for the CSC's. Whole CSC gave the normal increase in cell detachment which was unaltered with the addition of cAMP at any concentration (Figure 9.1, panel c). Both cell detachment and lysis did change however with the combination of vapour CSC and cAMP. There was an increase in more toxic injury of cell lysis with increasing cAMP concentrations and a concurrent decrease in cell detachment (Figure 9.1, panel c).

Protocol of Activation by Forskolin:- A549 epithelial cells were prepared for the detachment assay and on the morning of the experiment the plates were washed twice with PBS (200 μ l well⁻¹). Forskolin at 25 μ M (100 μ l aliquots) was added to the plates along with the MEM controls and the plates were then incubated for 30 minutes at 37°C, 5% CO₂. Thereafter, the aqueous CSC's were added (100 μ l aliquots) at twice the normal concentration which gave a final concentration of 10% in the wells. These plates were subsequently incubated for the 4 hours necessary for the detachment assay.

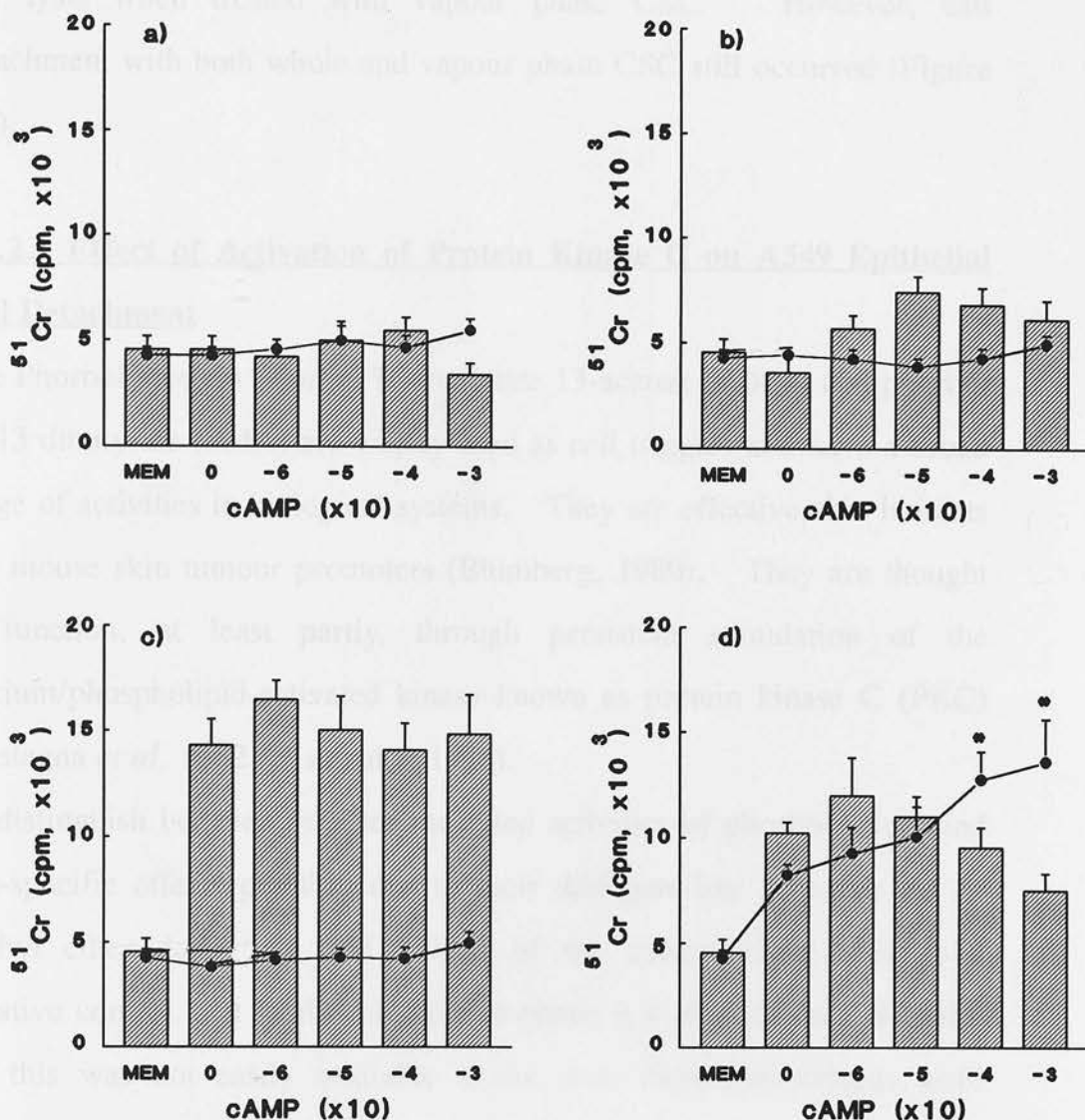


Figure 9.1. The effect of 30 minutes pre-incubation of cAMP in increasing concentration on a) MEM, b) 10% HBSS, c) 10% whole CSC, and d) 10% vapour CSC induced detachment (histograms) and lysis (\bullet) of A549 epithelial cells, measured in ^{51}Cr cpm. The histograms/ \bullet represent the mean of triplicate wells in four separate experiments and the bars indicate 1 SEM in each case. * $p < 0.05$; when compared with CSC alone (0).

Result:- Cells that had been pre-incubated with forskolin did not undergo cell lysis when treated with vapour phase CSC. However, cell detachment with both whole and vapour phase CSC still occurred (Figure 9.2).

9.1.2. Effect of Activation of Protein Kinase C on A549 Epithelial Cell Detachment

The Phorbol diesters Phorbol 12-myristate 13-acetate (PDBu) and phorbol 12-13 dibutyrate (PMA) are widely used as cell triggers and have a broad range of activities in biological systems. They are effective skin irritants and mouse skin tumour promoters (Blumberg, 1980). They are thought to function, at least partly, through persistent stimulation of the calcium/phospholipid-activated kinase known as protein kinase C (PKC) (Castagna *et al*, 1982; Nishizuka, 1984).

To distinguish between receptor-mediated activities of phorbol esters and non-specific effects possibly due to their detergent-like structure the 4-methyl ether derivative (PMA-4ME) of the compound is used as a negative control. It is also possible to obtain a 4-methyl ether of PDBu but this was not easily available at the time these experiments were carried out.

Protocol:- Both PDBu and PMA ($0.1, 1$ and $10\mu\text{gml}^{-1}$) were incubated with A549 epithelial cells in the detachment assay at 37°C , $5\% \text{CO}_2$ for 4 hours, along with negative control of PMA-4ME ($0.1, 1$ and $10\mu\text{gml}^{-1}$).

Results:- Significant epithelial detachment ($p < 0.05$) occurred with the addition of these PKC activators PDBu and PMA at $10\mu\text{gml}^{-1}$ (Figure 9.3, panel c & d), although cell lysis was not observed. There was an

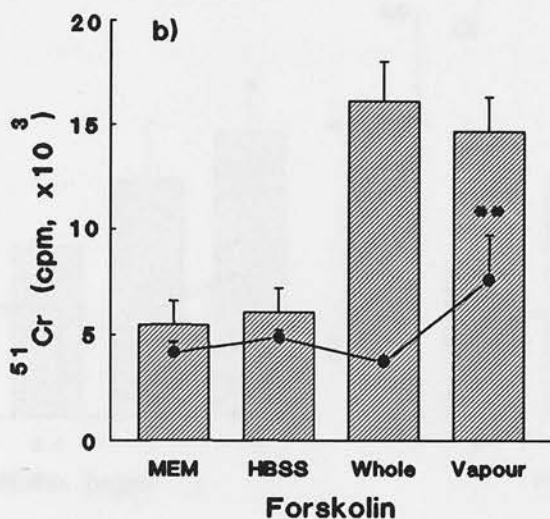
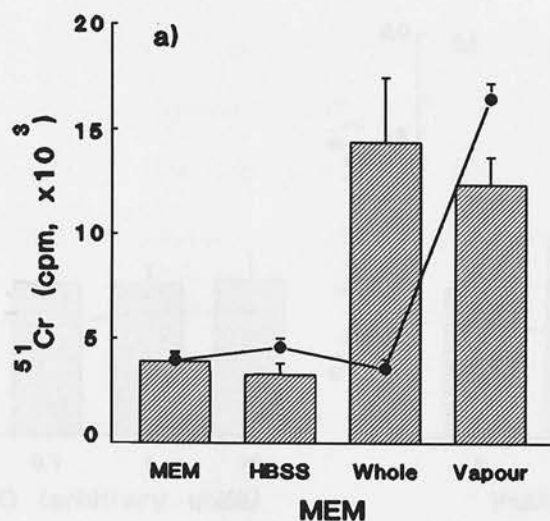


Figure 9.2. The effect of 2 hours pre-incubation of a) MEM and b) Forskolin on the detachment (histograms) and lysis (\bullet) of A549 epithelial cells induced by 10% whole or vapour CSC, measured in ^{51}Cr cpm. The histograms/ \bullet represent the mean of triplicate wells in four separate experiments and the bars indicate 1 SEM in each case. ** $p < 0.01$ compared with CSC alone.

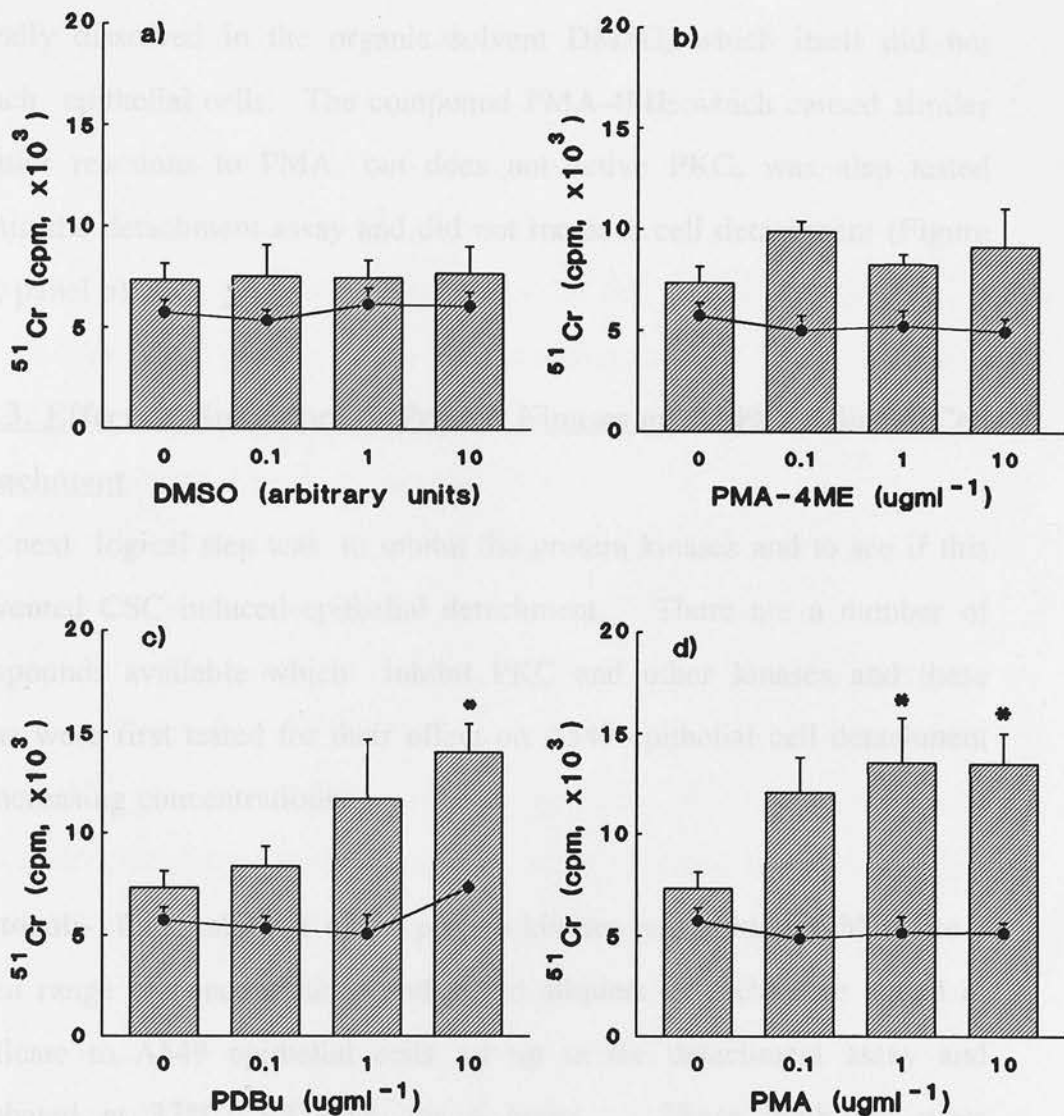


Figure 9.3. The effect of increasing concentrations of a) DMSO, b) PMA-4ME, c) PDBu, and d) PMA on the detachment (histograms) and lysis (●) of A549 epithelial cells, measured in ^{51}Cr cpm. The DMSO is in arbitrary units which are equivalent units to the concentration required to dissolve the other compounds. The histograms/● represent the mean of triplicate wells of three separate experiments and the bars indicate 1 SEM in each case. * $p < 0.05$ compared with medium alone (0).

apparent rise in cell detachment with PMA treated cells although this was not significant in a concentration of $0.1\mu\text{gml}^{-1}$. Both activators were initially dissolved in the organic solvent DMSO, which itself did not detach epithelial cells. The compound PMA-4ME which caused similar cellular reactions to PMA, but does not active PKC, was also tested within the detachment assay and did not increase cell detachment (Figure 9.3, panel b).

9.1.3. Effects of Inhibitors of Protein Kinases on A549 Epithelial Cell Detachment

The next logical step was to inhibit the protein kinases and to see if this prevented CSC induced epithelial detachment. There are a number of compounds available which inhibit PKC and other kinases and these alone were first tested for their effect on A549 epithelial cell detachment in increasing concentrations.

Protocol:- Each inhibitor of the protein kinases was diluted in MEM to a given range of concentrations and $200\mu\text{l}$ aliquots of each were added in triplicate to A549 epithelial cells set up in the detachment assay and incubated at 37°C , 5% CO_2 for 4 hours. These inhibitors were staurosporine (0.1,0.2,0.3,0.4,0.5,1,2 and $5\mu\text{M}$) calphostin C (10,50,100 and $1000\mu\text{M}$, (Kobayashi *et al*, 1989), 1-(5-isoquendolinesulfonyl)-2-methyl-piperazine, diHCl, otherwise known as H-7 (1,10,50 and $100\mu\text{M}$, (Kawamoto & Hidaka, 1984) and H-8 {N-[2-(methyl-amino)-ethyl]5-isoquinoline-sulfonamide, diHCl} (1,10, 50 and $100\mu\text{M}$, (Hidaka *et al*, 1984); all of which are potential inhibitors of PKC, although these compounds can also inhibit other kinases. Calphostin C required to be initially dissolved in DMSO and this was also tested for its potency in the

A549 epithelial detachment assay, using the appropriate concentrations.

Results:- One of the most widely utilised of these compounds is staurosporine (Tamaoki *et al*, 1986), which, when added at increasing concentrations (0.1 - 5 μ M), was observed to cause corresponding significant increases in A549 epithelial cell detachment (Figure 9.4). Even at the inhibitory concentration of 200nm, which has been used successfully by other workers (Schatz-Munding & Ullrich, 1992), epithelial detachment was observed. The compound was rejected for further experiments based on this evidence.

Calphostin C, H-7 or H-8 did not induce cell lysis in the range of concentrations tested when compared with control values (Figure 9.5). The highest concentration of calphostin C caused greater cell detachment than the control cells, with the lower doses producing no significant effect (Figure 9.5, panel b). H-7 did not cause epithelial cell detachment at any of the concentrations tested (Figure 9.5, panel c) whilst H-8 gave greater cell detachment at the higher concentrations (Figure 9.5, panel d). Again, the organic solvent (DMSO) used to dissolve calphostin C did not give rise to any change in epithelial detachment or lysis.

Another protein kinase inhibitor used was Sphingosine and this compound had to be dissolved in chloroform with gentle heating. However, when diluted in medium the Sphingosine precipitated out; this compound was also deemed unsuitable for use in the detachment assay.

9.1.4. Effect of Inhibition of Protein Kinases on Aqueous CSC

The ability of inhibitors of protein kinases to prevent the cell detachment caused by aqueous CSC was examined using the following protocol.

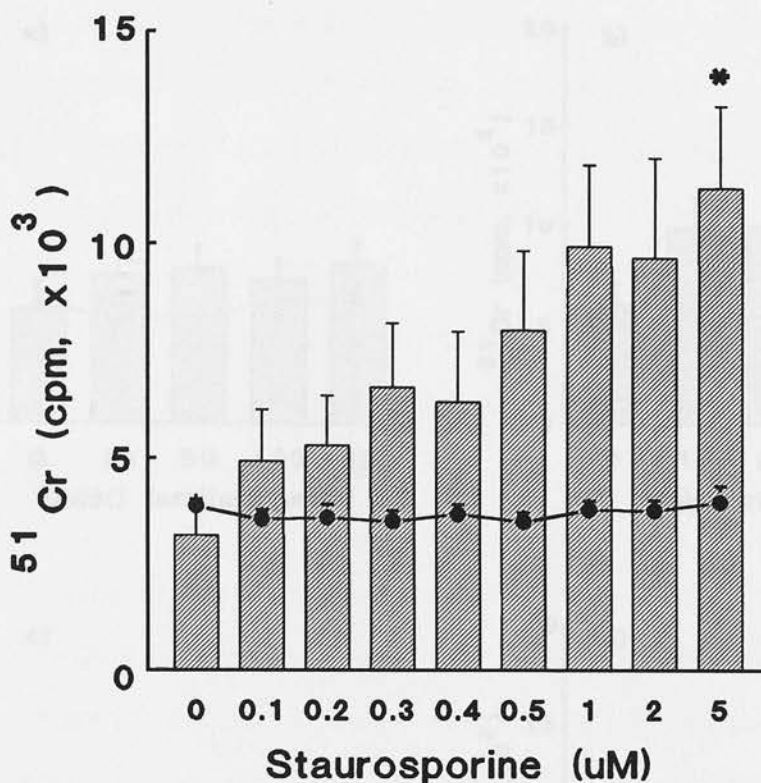


Figure 9.4. The effect of increasing concentrations of the PKC inhibitor Staurosporine on the detachment (histograms) and lysis (●) of A549 epithelial cells, measured in ⁵¹Cr cpm. The histograms/● represent the mean of triplicate wells in four separate experiments and the bars indicate 1 SEM in each case. * p < 0.05 compared with medium alone (0).

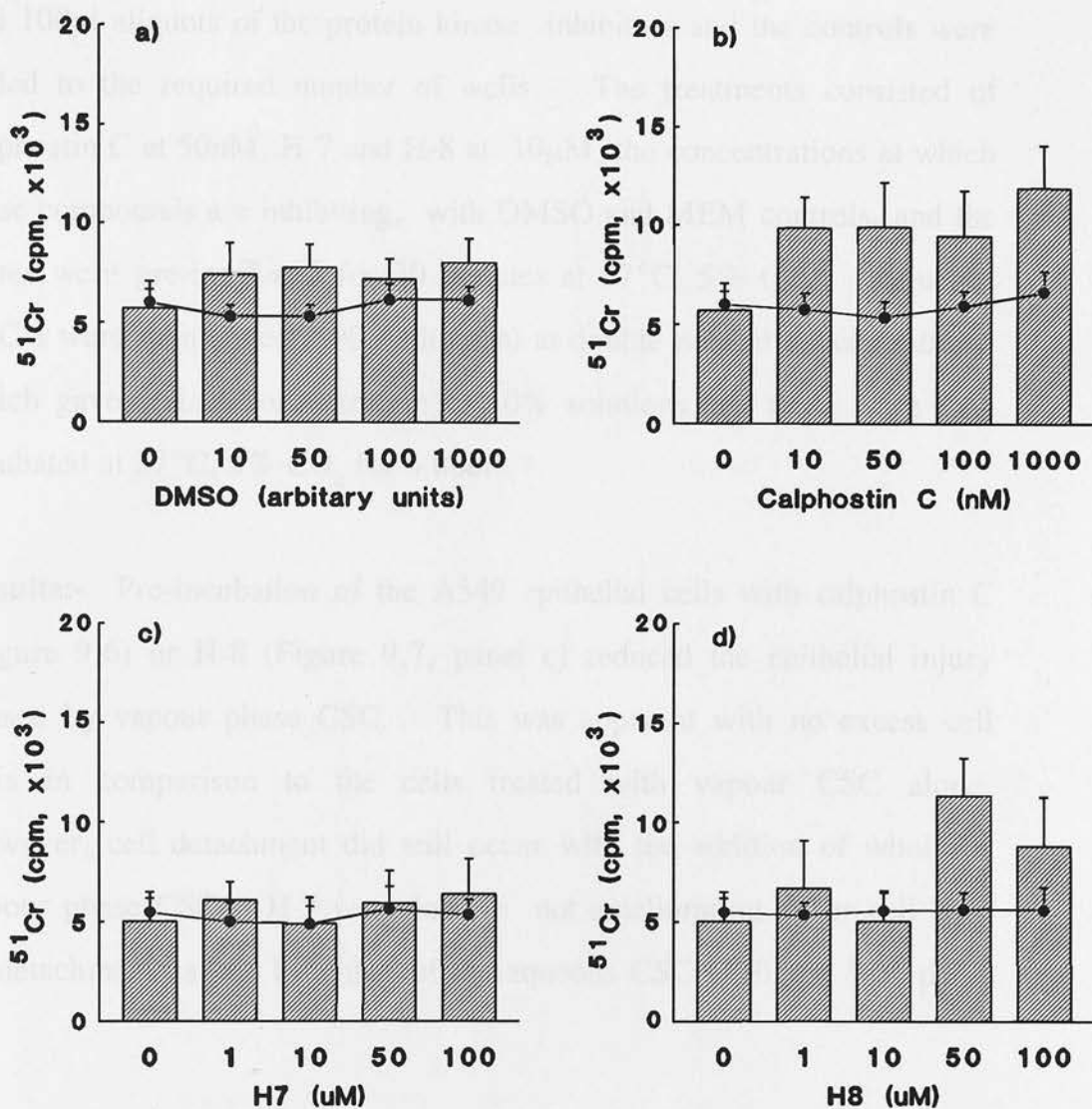


Figure 9.5. The effect of increasing concentrations of the protein kinase inhibitors a) DMSO, b) Calphostin C, c) H7, and d) H8 on the detachment (histograms) and lysis (\bullet) of A549 epithelial cells measured in ^{51}Cr cpm. The DMSO is in arbitrary units which are equivalent to the concentrations of calphostin C. The histograms/ \bullet represent the mean of three separate experiments and the bars indicate 1 SEM in each case.

Protocol:- The A549 epithelial cells were prepared for a detachment assay. After overnight incubation, the cells were washed twice with PBS and 100 μ l aliquots of the protein kinase inhibitors and the controls were added to the required number of wells. The treatments consisted of calphostin C at 50nM, H-7 and H-8 at 10 μ M, the concentrations at which these compounds are inhibiting, with DMSO and MEM controls, and the plates were pre-incubated for 30 minutes at 37°C, 5% CO₂. Aqueous CSC's were then added (100 μ l aliquots) at double normal concentrations, which gave a final concentration of 10% solutions and these were then incubated at 37°C, 5% CO₂ for 4 hours.

Results:- Pre-incubation of the A549 epithelial cells with calphostin C (Figure 9.6) or H-8 (Figure 9.7, panel c) reduced the epithelial injury caused by vapour phase CSC. This was apparent with no excess cell lysis in comparison to the cells treated with vapour CSC alone. However, cell detachment did still occur with the addition of whole or vapour phase CSC. H-7 was alone in not ameliorating either cell lysis or detachment caused by either of the aqueous CSC's (Figure 9.7, panel b).

9.2. ROLE OF CALCIUM IN A549 EPITHELIAL CELL DETACHMENT

Changes in intracellular calcium levels reflect changes in cell activity. It is possible to approach this by a number of different ways, one by investigating the effect of increasing [Ca²⁺]_i levels on A549 epithelial cells and another by examination of the effect of inhibiting rises in [Ca²⁺]_i on aqueous CSC induced A549 epithelial cell detachment.

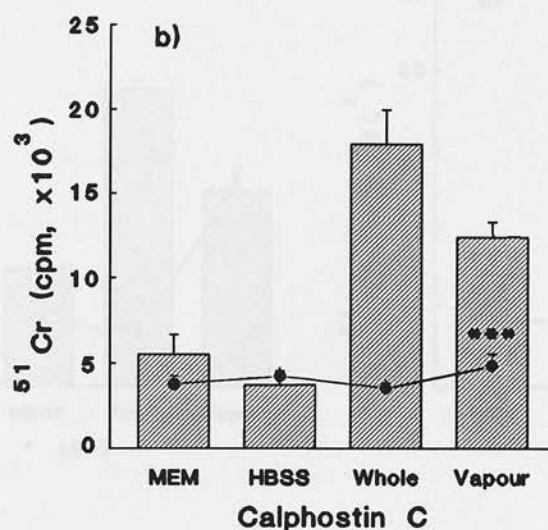
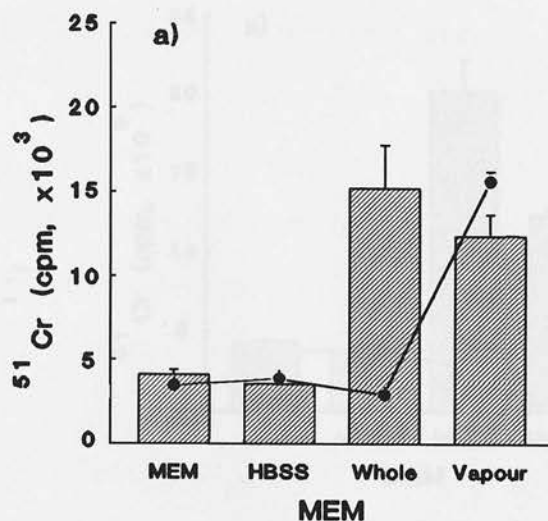


Figure 9.6. The effect of 2 hours pre-incubation in a) MEM, and b) calphostin C on the detachment (histograms) and lysis (\bullet) of A549 epithelial cells induced by 10% whole or vapour CSC, measured in ^{51}Cr cpm. The histograms/ \bullet represent the mean of triplicate wells in four separate experiments and the bars indicate 1 SEM in each case. *** $p < 0.001$ compared with CSC alone.

9.2.1 Effect of Alteration in Intracellular Calcium Stores

It is possible to alter the intracellular calcium stores of a cell using calcium ionophores. Two compounds that have been used to alter intracellular calcium stores are thapsigargin and A23187. Thapsigargin is a specific inhibitor of the endoplasmic reticulum Ca^{2+} -ATPase, which pumps Ca^{2+} from the cytosol into the endoplasmic reticulum. A23187 is a calcium ionophore that increases intracellular Ca^{2+} by forming a complex with Ca^{2+} and allowing it to pass through the cell membrane.

Protocol: Both these compounds were used in the following experiments. Cells were grown in MEM and then treated with either thapsigargin or A23187 for 2 hours. After washing the cells with MEM, they were treated with 10% whole CSC or 10% vapour CSC for 2 hours. The cells were then harvested and the ^{51}Cr cpm measured.

Results: The calcium ionophore A23187 had no effect on the detachment of A549 cells, but did produce an increase in ^{51}Cr release.

(Figure 9.7 panel a). In higher concentrations of the calcium ionophore

A23187 (10 μM), the detachment of A549 cells was increased (Figure 9.7 panel b).

Thapsigargin had no effect on the detachment of A549 cells, but did produce an increase in ^{51}Cr release.

(Figure 9.7 panel c). In higher concentrations of the calcium ionophore

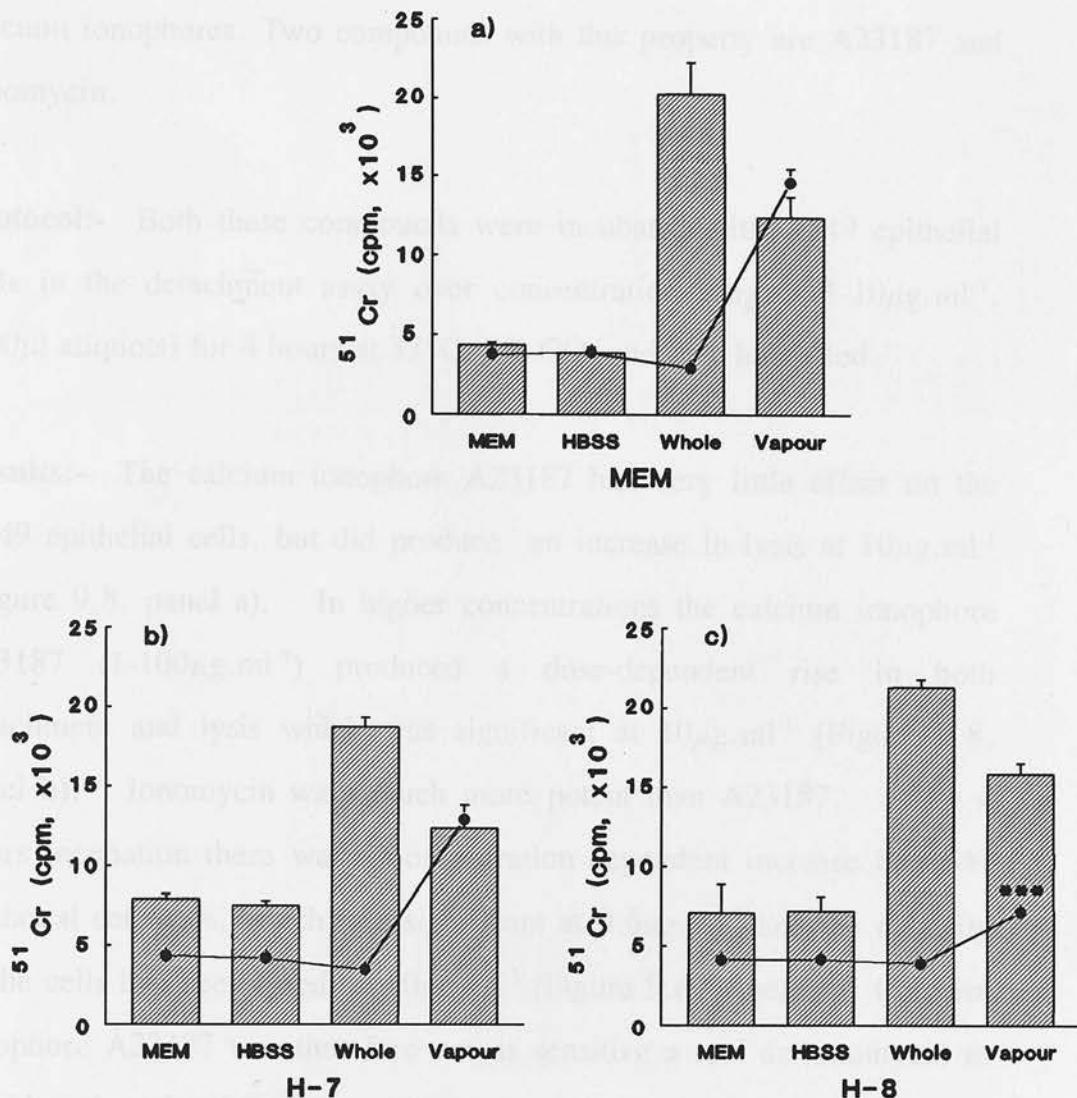


Figure 9.7. The effect of 2 hours pre-incubation in a) MEM, b) H-7, and c) H-8 on the detachment (histograms) and lysis (\bullet) of A549 epithelial cells induced by 10% whole or vapour CSC, measured in ^{51}Cr cpm. The histograms/ \bullet represent the mean of triplicate wells in three separate experiments and the bars indicate 1 SEM in each case. ** $p < 0.01$ compared with CSC alone.

9.2.1. Effect of Alteration in Intracellular Calcium Status

It is possible to alter the intracellular calcium status of a cell using calcium ionophores. Two compounds with this property are A23187 and ionomycin.

Protocol:- Both these compounds were incubated with A549 epithelial cells in the detachment assay over concentration range $0.1-10\mu\text{g.ml}^{-1}$. (200 μl aliquots) for 4 hours at 37°C , 5% CO_2 and then harvested.

Results:- The calcium ionophore A23187 had very little effect on the A549 epithelial cells, but did produce an increase in lysis at $10\mu\text{g.ml}^{-1}$ (Figure 9.8, panel a). In higher concentrations the calcium ionophore A23187 ($1-100\mu\text{g.ml}^{-1}$) produced a dose-dependent rise in both detachment and lysis which was significant at $10\mu\text{g.ml}^{-1}$ (Figure 9.8, panel b). Ionomycin was much more potent than A23187. After 4 hours incubation there was a concentration dependent increase in A549 epithelial cell lysis, which was significant at $0.6\mu\text{g.ml}^{-1}$ and the majority of the cells had been lysed by $10\mu\text{g.ml}^{-1}$ (Figure 9.6, panel c). Calcium ionophore A23187 was therefore not as sensitive a tool as ionomycin to elucidate the role of Ca^{2+} in epithelial detachment.

9.2.2. Time Course of A549 Epithelial Cell Detachment with Ionomycin

In Chapter 3 it was observed that A549 epithelial lysis with vapour CSC did not occur until after 2 hours incubation. To test whether this was calcium related a time course of A549 epithelial detachment was run with varying concentrations of ionomycin.

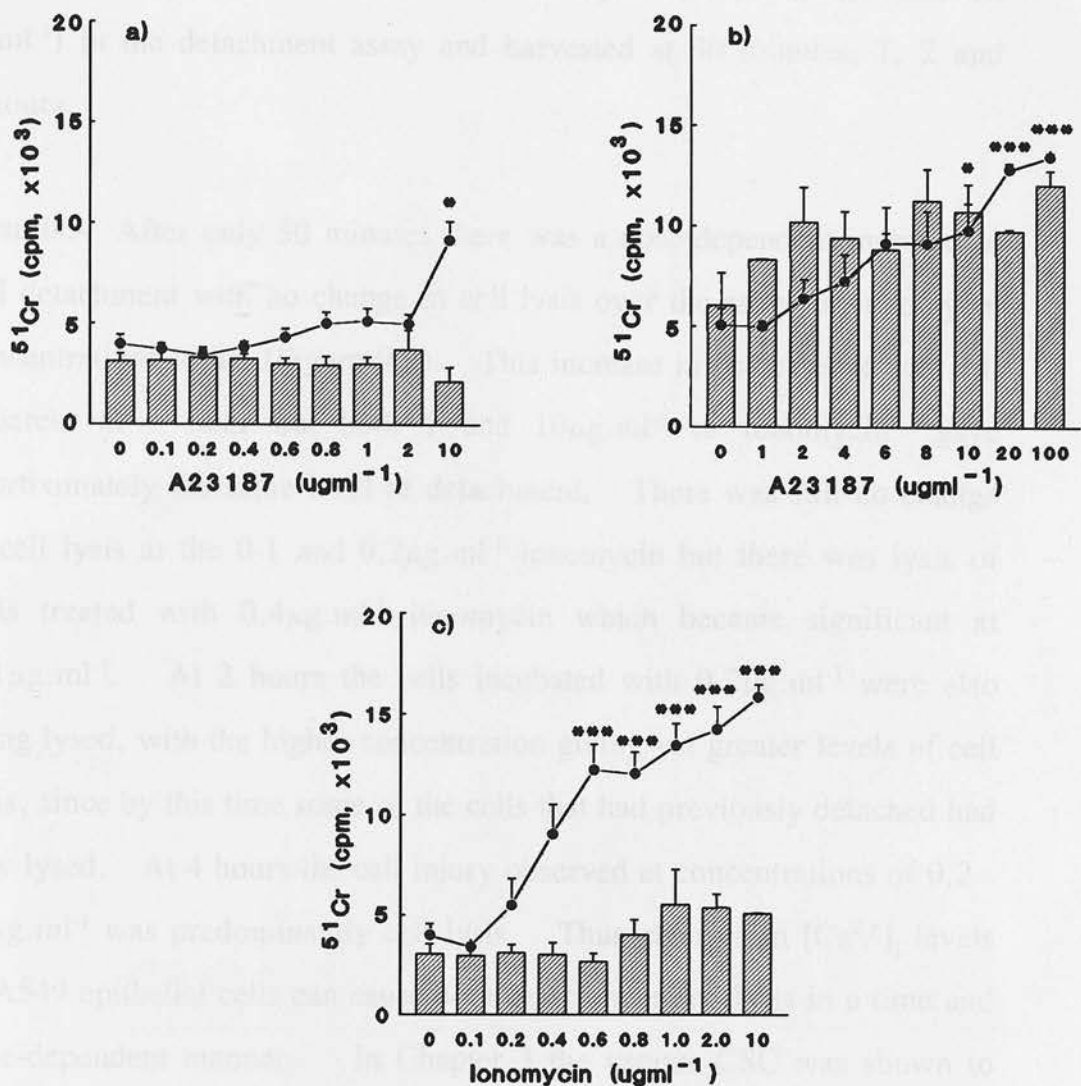


Figure 9.8. The effect of increasing concentrations of a) and b) A23187, and c) Ionomycin on detachment (histograms) and lysis (—●—) of A549 epithelial cells, measured in ⁵¹Cr cpm. The histograms/—●— represent a mean of triplicate wells in four separate experiments for a) and b), and six separate experiments for c) and the bars indicate 1 SEM. * p < 0.05; *** p < 0.001 when compared with medium alone (0).

Protocol:- The A549 epithelial cells were incubated at 37°C, 5% CO₂ with the selected concentrations of ionomycin (0.1,0.2,0.4,1 and 10 µgml⁻¹) in the detachment assay and harvested at 30 minutes, 1, 2 and 4 hours.

Results:- After only 30 minutes there was a dose-dependent increase in cell detachment with no change in cell lysis over the range of ionomycin concentrations tested (Figure 9.9). This increase in detachment was still apparent at 1 hour but both 1 and 10µg.ml⁻¹ of ionomycin gave approximately the same level of detachment. There was still no change in cell lysis at the 0.1 and 0.2µg.ml⁻¹ ionomycin but there was lysis of cells treated with 0.4µg.ml⁻¹ ionomycin which became significant at ≥1µg.ml⁻¹. At 2 hours the cells incubated with 0.2µg.ml⁻¹ were also being lysed, with the higher concentration giving yet greater levels of cell lysis, since by this time some of the cells that had previously detached had now lysed. At 4 hours the cell injury observed at concentrations of 0.2 - 10µg.ml⁻¹ was predominantly cell lysis. Thus changes in [Ca²⁺]_i levels of A549 epithelial cells can cause both detachment and lysis in a time and dose-dependent manner. In Chapter 3 the vapour CSC was shown to cause both cell detachment and lysis at 4 hours and it was not until later that the cell injury became predominantly cell lysis for this type of condensate. This does not however rule out areas for changes in [Ca²⁺]_i in either epithelial cell detachment or lysis caused by condensates. The next step was to try and establish the relationship between [Ca²⁺]_i levels in A549 epithelial cells and treatment with condensates.

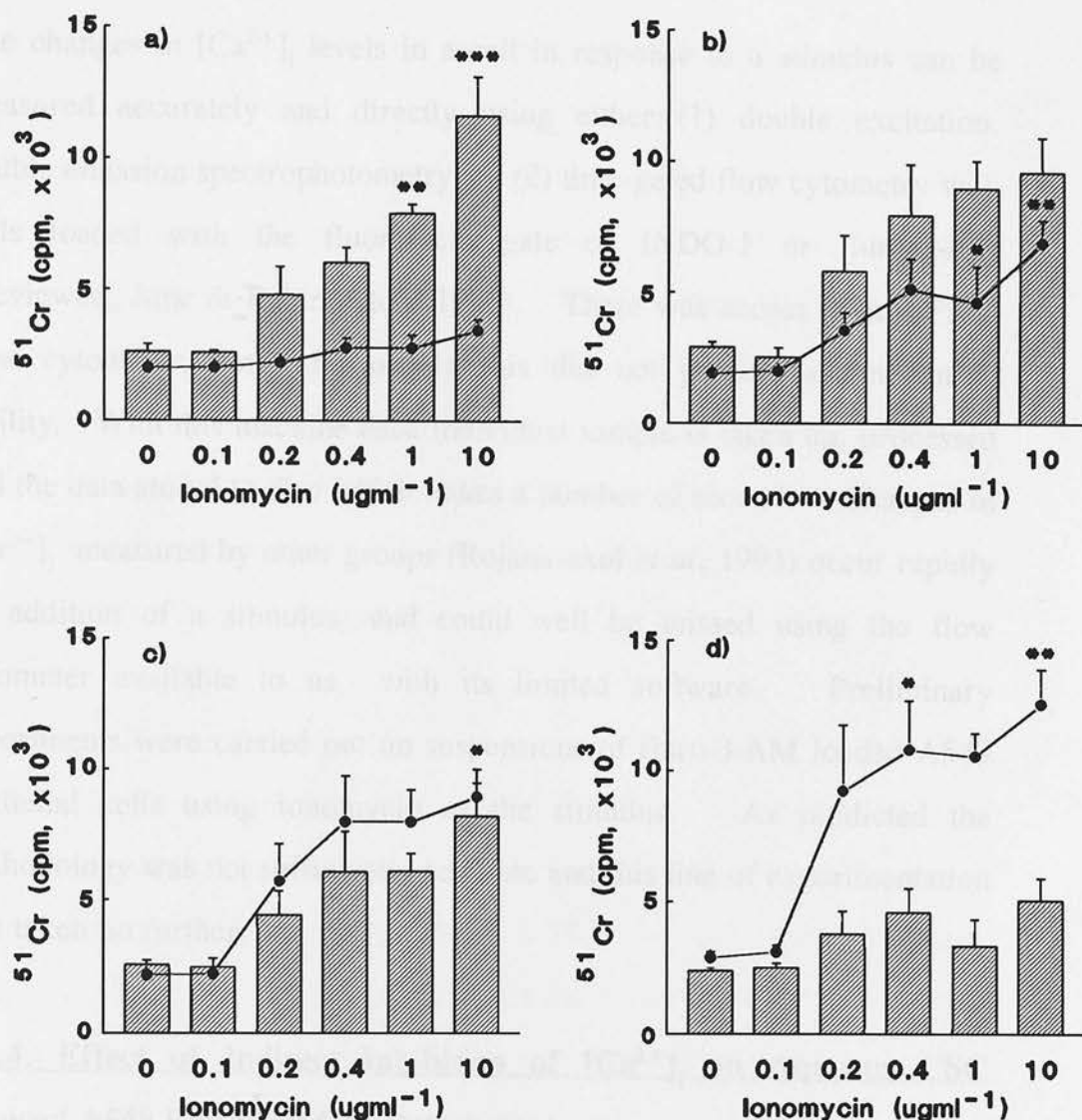


Figure 9.9. The effect of increasing concentrations of lonomycin at a) 30 minutes, b) 1 hour, c) 2 hours, and d) 4 hours on detachment (histograms) and lysis (\bullet) of A549 epithelial cells, measured in ^{51}Cr cpm. The histograms/ \bullet represent the mean of triplicate wells of five separate experiments and the bars indicate 1 SEM in each case. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; compared with alone (0).

9.2.3. Changes in $[Ca^{2+}]_i$ in A549 Epithelial Cells Due to Aqueous CSC

The changes in $[Ca^{2+}]_i$ levels in a cell in response to a stimulus can be measured accurately and directly using either (1) double excitation, double emission spectrophotometry or (2) time-gated flow cytometry with cells loaded with the fluorescent dye of INDO-1 or fluoro-3-AM (Reviewed, June & Rabinovitch, 1988). There was access to an EPICS flow cytometer, but unfortunately this did not possess a time-gating facility. With this machine each individual sample is taken up, processed and the data stored to disc which takes a number of seconds. Changes in $[Ca^{2+}]_i$ measured by other groups (Rojanasakul *et al*, 1993) occur rapidly on addition of a stimulus, and could well be missed using the flow cytometer available to us with its limited software. Preliminary experiments were carried out on suspensions of fluoro-3-AM loaded A549 epithelial cells using ionomycin as the stimulus. As predicted the methodology was not sufficiently accurate and this line of experimentation was taken no further.

9.2.4. Effect of Indirect Inhibition of $[Ca^{2+}]_i$ on Aqueous CSC Induced A549 Epithelial Cell Detachment

The alternative indirect method was to chelate the extracellular Ca^{2+} , so inhibiting the $[Ca^{2+}]_i$ response and hopefully in the case of CSC reducing the level of cell injury. A common Ca^{2+} chelator is EDTA, while EGTA chelates both Ca^{2+} and Mg^{2+} . These compounds used in low concentrations will chelate the ions without affecting the cells *per sé*, but at a higher concentration will interfere with the cell surface integrins, leading to cell detachment and at even higher concentrations become toxic leading to cell lysis (discussed in Chapter 7). The concentration of the

chelator used in these experiments was therefore critical. Based on the experiments in Chapter 7 a concentration range of 0.1 - 10 μ g.ml⁻¹ of both EDTA and EGTA was used in the following protocol.

Protocol:- Epithelial cells were prepared in the usual way for the detachment assay. On the morning of the experiment the cells were washed twice with PBS and 100 μ l aliquots of either EDTA or EGTA were added in increasing concentrations (0.1, 0.5, 1, 2 & 10 μ M) to the wells, as well as the appropriate controls. After a 2 hour incubation period 100 μ l of double concentration CSC were added to the cells, this giving a final concentration of 10% for the CSC. The plates were then incubated for a further 4 hours at 37°C, 5%CO₂.

Results:- The chelators conferred a degree of protection to the A549 epithelial cells treated with both types of CSC. There was less cell detachment caused by whole CSC with both EDTA (Figure 9.10) and EGTA (Figure 9.11) pre-treatment. EGTA at 10 μ M was particularly potent in preventing detachment with only a small rise over background. The cell lysis caused by vapour CSC on A549 epithelial cells was also reduced with pre-treatment by EDTA and EGTA in the higher concentrations. This data suggests a role for calcium in the CSC induced A549 epithelial cell damage in vitro.

9.3. ROLE OF G-PROTEIN IN A549 EPITHELIAL CELL DETACHMENT

Since G-proteins are important in regulating a large part of the intracellular signalling system, it is possible that inhibition of these proteins will effect CSC induced epithelial cell detachment.

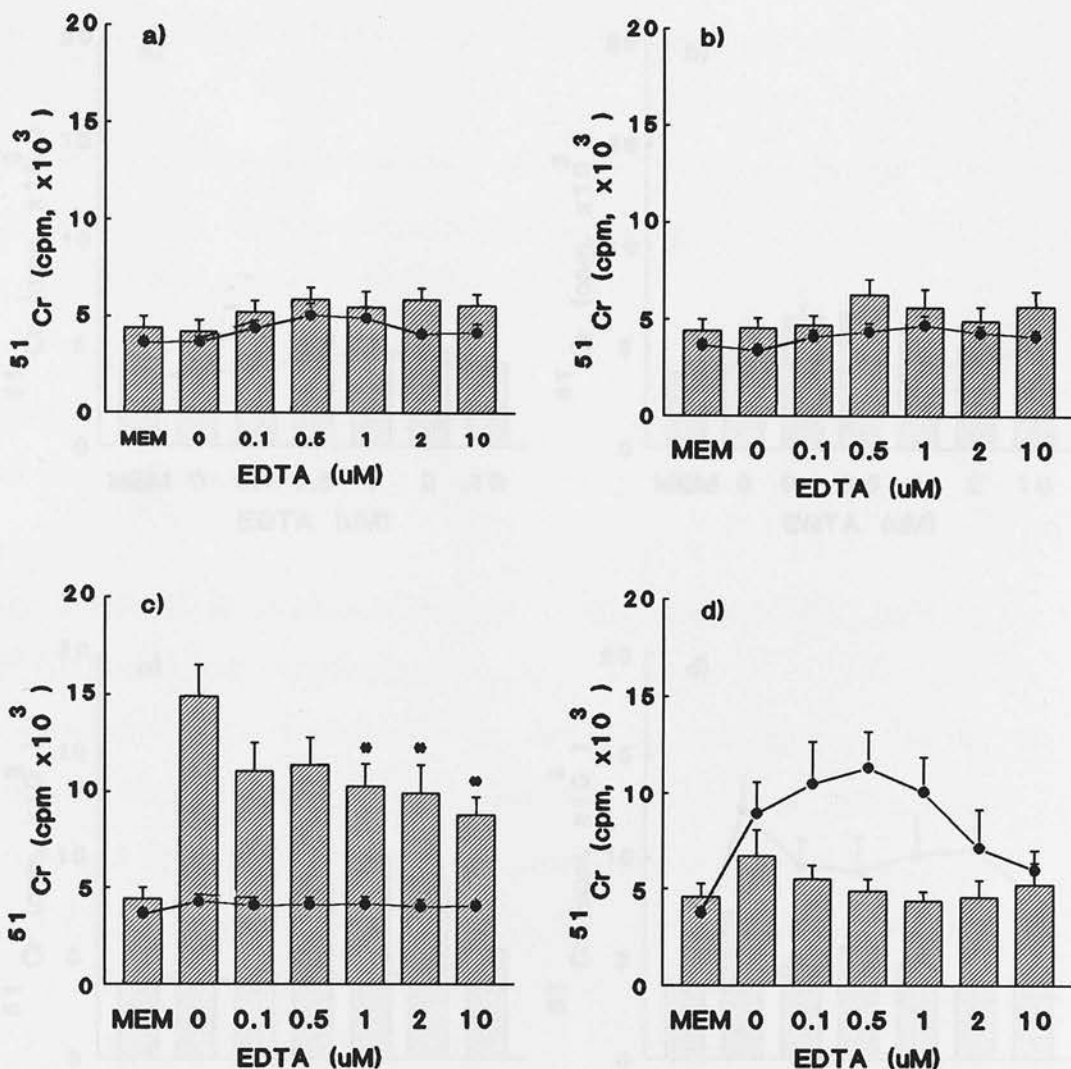


Figure 9.10. The effect of 2 hours pre-incubation of increasing concentrations of EDTA on a) MEM, b) 10% HBSS c) 10% whole CSC, and d) 10% vapour CSC induced detachment (histograms) and lysis (\bullet) of A549 epithelial cells, measured in ^{51}Cr cpm. The histograms/ \bullet represent the mean of triplicate wells of five separate experiments and the bars indicate 1 SEM in each case. * $p < 0.05$ compared with CSC alone (0).

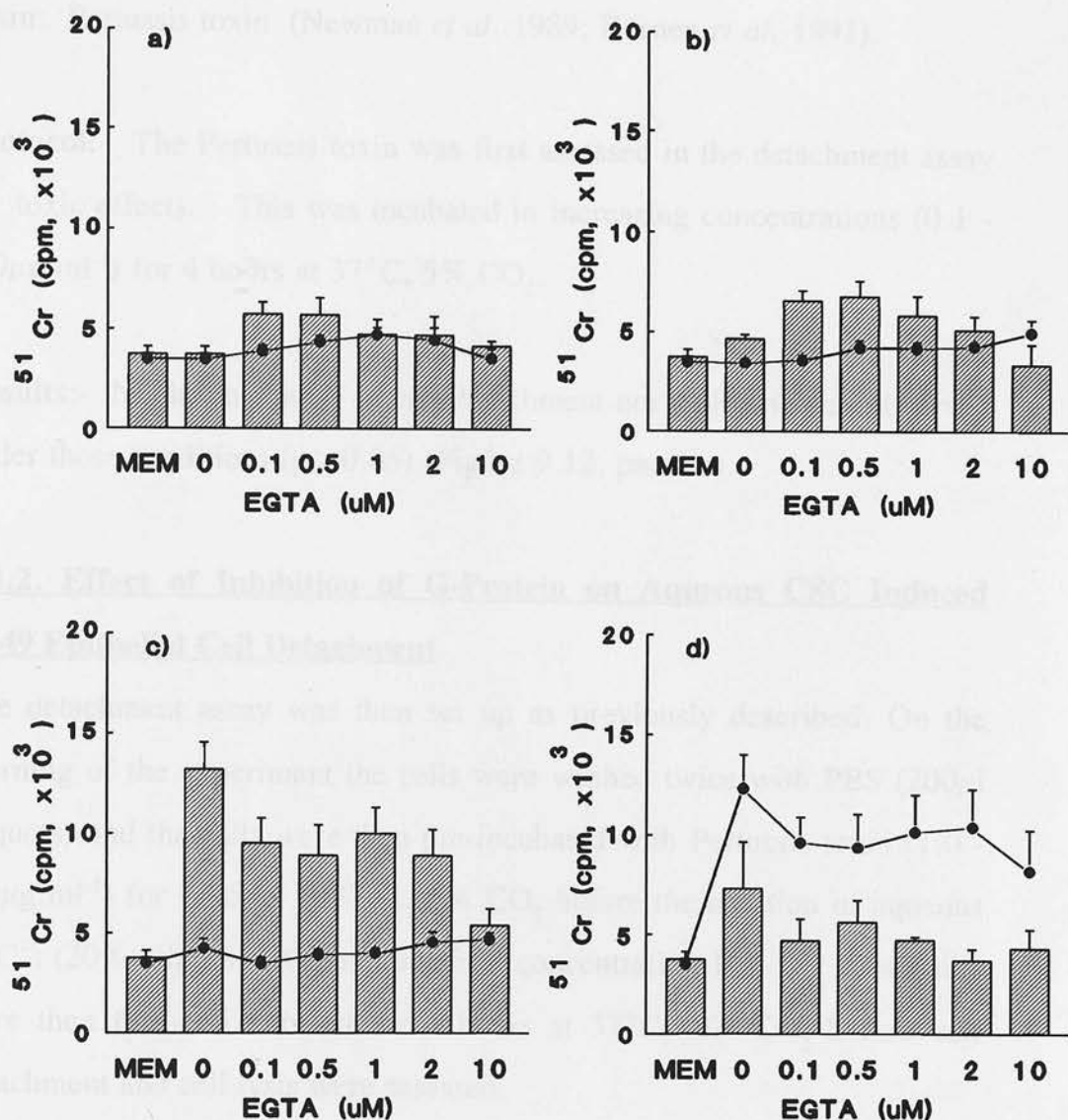


Figure 9.11. The effect of 2 hour pre-incubation of increasing concentrations of EGTA on a) MEM, b) 10% HBSS, c) 10% whole CSC, and d) 10% vapour CSC induced detachment (histograms) and lysis (\bullet) of A549 epithelial cells, measured in ^{51}Cr cpm. The histograms/ \bullet represent the mean of triplicate wells in 5 separate experiments and the bars indicate 1 SEM in each case.

9.3.1. Effect of Pertussis Toxin on A549 Epithelial Cell Detachment

It is possible to inhibit the function of some G-proteins using the bacteria toxin: Pertussis toxin (Newman *et al*, 1989; Kernen *et al*, 1991).

Protocol:- The Pertussis toxin was first assessed in the detachment assay for toxic effects. This was incubated in increasing concentrations (0.1 - 5.0 μ g.ml⁻¹) for 4 hours at 37°C, 5% CO₂.

Results:- Neither a change in cell detachment nor cell lysis was observed under these conditions ($p > 0.05$) (Figure 9.12, panel a).

9.3.2. Effect of Inhibition of G-Protein on Aqueous CSC Induced A549 Epithelial Cell Detachment

The detachment assay was then set up as previously described. On the morning of the experiment the cells were washed twice with PBS (200 μ l aliquots) and the cells were then pre-incubated with Pertussis toxin (1.0 - 5.0 μ g.ml⁻¹) for 2 hours at 37°C, 5% CO₂ before the addition of aqueous CSC's (20% dilution 100 μ l aliquot final concentration 10%). These cells were then further incubated for 4 hours at 37°C, 5% CO₂ before cell detachment and cell lysis were assessed.

Results:- The A549 epithelial cells were unaffected by pretreatment with Pertussis toxin and then, with either MEM or 10% HBSS solution showed no adverse effects to this treatment. The Pertussis toxin pre-treatment did not prevent the A549 epithelial cell injury caused by whole CSC but did partially protect against the vapour CSC induced cell lysis. There was reduced cell lysis with a concurrent increase in the cell detachment (Figure 9.12, panel d).

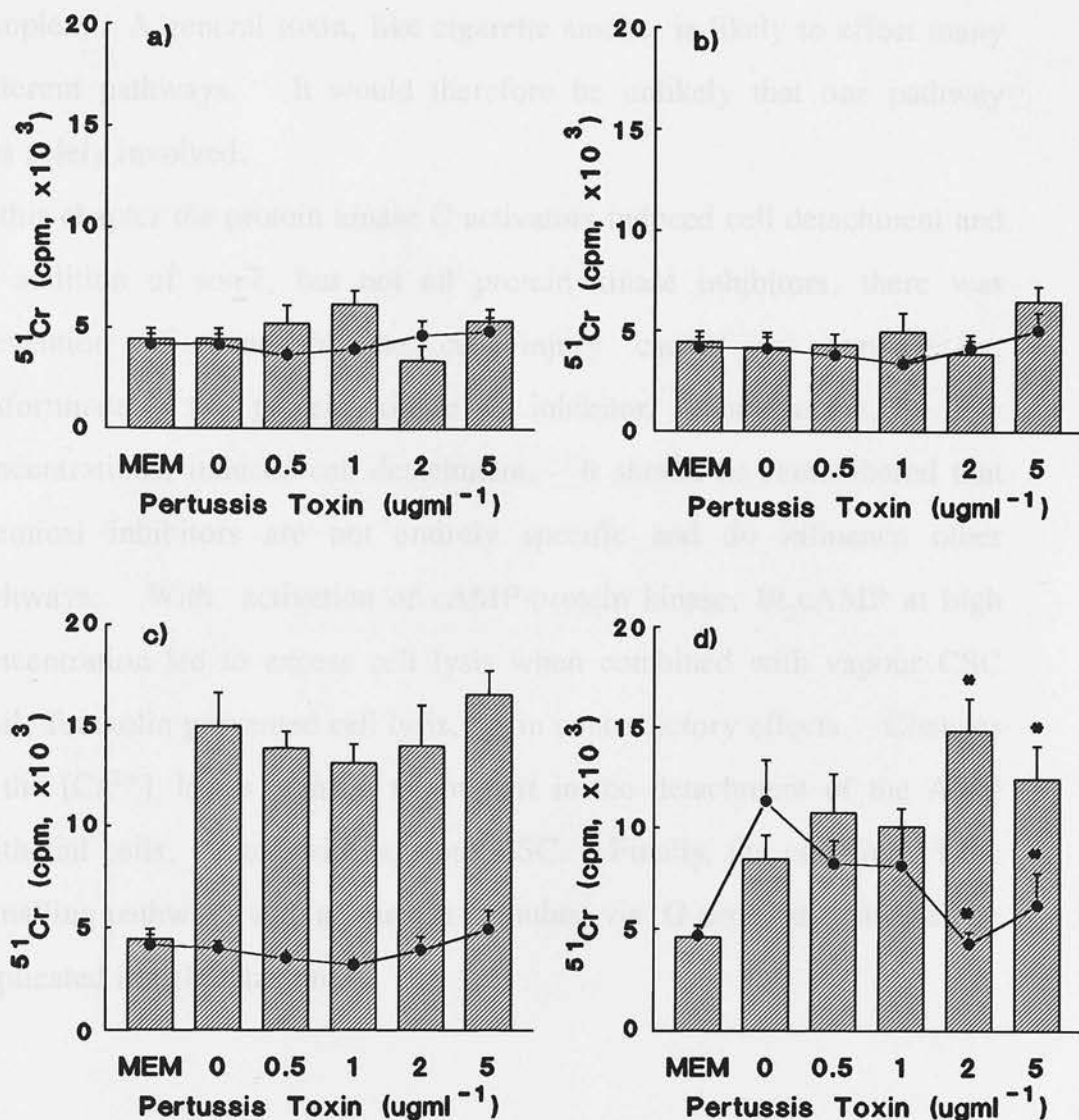


Figure 9.12. The effect of 2 hours pre-incubation of Pertussis toxin on a) MEM, b) 10% HBSS, c) 10% whole CSC, and d) 10% vapour CSC induced detachment (histograms) and lysis (\bullet) of A549 epithelial cells, measured in ^{51}Cr cpm. The histograms/ \bullet represent the mean of triplicate wells of three separate experiments and the bars indicate 1 SEM in each case. * $p < 0.05$; ** $p < 0.01$ compared with CSC alone (0).

SUMMARY

The pathways of intracellular communication are intertwined and complex. A general toxin, like cigarette smoke, is likely to effect many different pathways. It would therefore be unlikely that one pathway was solely involved.

In this chapter the protein kinase C activators induced cell detachment and on addition of some, but not all protein kinase inhibitors, there was prevention of some of the cell injury caused by condensates. Unfortunately, the protein kinase C inhibitor, staurosporine, at low concentrations, induced cell detachment. It should be remembered that chemical inhibitors are not entirely specific and do influence other pathways. With activation of cAMP-protein kinase, Bt_2cAMP at high concentration led to excess cell lysis when combined with vapour CSC while forskolin prevented cell lysis, again contradictory effects. Changes in the $[Ca^{2+}]_i$ levels play a major part in the detachment of the A549 epithelial cells, treated with aqueous CSC. Finally, the coupling of the signalling pathways with an outside stimulus, via G proteins, may well be implicated in cell detachment.

CHAPTER 10

GENERAL DISCUSSION

Epithelial cells are important in maintaining the integrity and fluid balance of tissues (Foulds & Fawcett, 1984). Injury to the epithelium may be an important early event following exposure to cigarette smoke and other oxidant gases, manifesting as an increase in epithelial permeability in the airspaces. A partial or total loss of the airway epithelium after a single 1 hour exposure to cigarette smoke by inhalation has been reported by Lam (1986).

CHAPTER 10

GENERAL DISCUSSION

Following smoke-induced injury to type I alveolar epithelial cells there may be modified type II cell hyperplasia. This occurs as an early event following exposure to oxidant gases such as ozone and NO₂ (Wright *et al.*, 1987) which are components of cigarette smoke. Chronic smoke has been shown to damage both type I (Burns *et al.*, 1985) and type II pneumocytes (Le Marquand *et al.*, 1981) in vivo.

CHOICE OF INDICATOR CELLS FOR THE STUDY

In vitro investigations involving the alveolar epithelium have centred around the type II pneumocyte as isolation of type I pneumocytes has proved to be impossible. There are a limited number of papers published on the isolation of human type II pneumocytes (Feldman *et al.*, 1984; Hargis *et al.*, 1989). These cell populations are not completely pure, since they contain alveolar macrophages, fibroblasts or endothelial cells, which could influence any ways in which the type II pneumocyte were

CHAPTER 10

GENERAL DISCUSSION

Epithelial cells are important in maintaining the integrity and fluid balance of tissue (Hoidal & Niewoehner, 1983). Injury to the epithelium may be an important early event following exposure to cigarette smoke and other oxidant gases, manifesting as an increase in epithelial permeability in the airspaces. A partial or total loss of epithelium of the rat larynx after a single 1 hour exposure to cigarette smoke by inhalation has been reported by Lam (1980). This was followed by rapid regeneration of the epithelium with the subsequent development of hyperplasia. Following smoke-induced injury to type I alveolar epithelial cells there may be marked type II cell hyperplasia. This occurs as an early event following exposure to oxidant gases such as ozone and NO₂ (Wright *et al*, 1987) which are components of cigarette smoke. Cigarette smoke has been shown to damage both type I (Burns *et al*, 1989) and type II pneumocytes (Le Mesurier *et al*, 1981) in vivo.

CHOICE OF INDICATOR CELLS FOR THE STUDY

In vitro investigations involving the alveolar epithelium have centred around the type II pneumocyte as isolation of type I pneumocytes has proved to be impossible. There are a limited number of papers published on the isolation of human type II pneumocytes (Robinson *et al*, 1984; Bingle *et al*, 1990). These cell populations are not completely pure, since they contain alveolar macrophages, fibroblasts or endothelial cells, which could influence any assays to which the type II pneumocyte were

subjected. The majority of investigations published on alveolar pneumocytes have either involved primary culture of type II cells from non-human tissue (Buckley *et al*, 1987) or cell lines (Cantin *et al*, 1993). In this thesis an alveolar epithelial cell line and a bronchial epithelial cell line were utilised as models to study the injurious effects of cigarette smoke (Leiber *et al*, 1976; Reddel, 1988). This allowed the use of two types of epithelial cells derived from human lung tissue, which produced pure cultures and cells that supposedly do not vary between experiments. A limited number of experiments were also carried out on freshly isolated rat type II pneumocytes. Human type II pneumocytes were not used because of availability of tissue and the lack of purity of the final cell suspension, these reasons are discussed in greater detail in Section 7. The assays of epithelial cell attachment, detachment and proliferation were used to model the adverse effects of cigarette smoke on the integrity of the pulmonary epithelial barrier, which might underlie the increased epithelial permeability found in smokers. Epithelial injury may also be a factor in the development of the terminal bronchiolitis (Niewoehner *et al*, 1974) and increased permeability (Minty *et al*, 1981) that are a feature of the lungs in asymptomatic smokers.

The results of these studies show that fresh, whole cigarette smoke and vapour phase cigarette smoke, together with their condensates, have a profound, adverse effect on the attachment, detachment and proliferation of epithelial cells in vitro. These effects may be mediated by oxidants in cigarette smoke since the non-enzymatic anti-oxidant, reduced glutathione protected against the effect, and the oxidant H_2O_2 mimicked the effect of cigarette smoke. Bridges (1985) found that whole or vapour phase cigarette smoke and their water-soluble fraction (WSF) of cigarette smoke condensate had no effect on cell viability as determined by trypan blue

exclusion and enzyme release. With the cigarette smoke exposure used in this thesis the viability of the A549 epithelial cells as assessed by trypan blue exclusion immediately after exposure was also found to be consistently >95%.

EPITHELIAL INJURY

Oxidant-induced epithelial cell injury has been studied in experiments using ^{51}Cr -labelled tracheal explants. In this model the addition of both O_2^- and H_2O_2 (10-300 μM) (Martin *et al*, 1981) resulted in the release of lactate dehydrogenase (LDH) from the explant, indicating epithelial cell injury. However, Buckley and co-workers (1987) have suggested that cell detachment is an earlier and more sensitive indicator of oxidant injury than the release of LDH. Furthermore, Chopra *et al* (1987) have also shown ^{51}Cr release to be a more sensitive indicator of cell injury than release of either LDH or ^{111}In label. Buckley *et al* (1987) have also used the release of ^{51}Cr label into the culture medium as a marker of cytotoxicity, a technique which is comparable with the measurement of cell lysis in this study. Epithelial detachment and decreased proliferation can be oxidant mediated. Evidence for this can be found in the non-lytic injury which could be induced in A549 epithelial cells in culture by exogenous H_2O_2 in low concentrations (<15 μM), whereas higher concentrations (>15 μM) produced lytic injury. The BEA-2B epithelial cell line treated with the lower concentrations of H_2O_2 (1-15 μM) also gave predominantly cell detachment with low levels of cell lysis, whereas the higher concentrations of H_2O_2 led to extensive cell lysis. Similar oxidant-induced cell injury has also been observed following exposure of rat type II epithelial cells (Buckley *et al*, 1987) and endothelial cells (Ager & Gordon, 1984) to H_2O_2 .

In the studies reported here, vapour phase CSC was more toxic to the A549 epithelial cells, producing both a detachment and a lytic injury, whereas whole CSC produced only detachment in A549 epithelial cells. The vapour CSC was also more toxic to the BEA-2B epithelial cells than the whole CSC, although the whole CSC produced cell lysis at the top concentrations. The BEA-2B epithelial cells were more susceptible to injury than A549 cells. Although this was not further investigated biochemically, this difference in susceptibility is of interest. Difference in toxicity between whole cigarette smoke and vapour phase cigarette smoke has been observed by other groups (Laurent *et al*, 1983; Pryor & Dooley, 1985). It has been suggested that the carbon particles in whole smoke cigarette can absorb a proportion of toxins present, including oxidants, making them unavailable for cell injury (Kilburn & McKenzie, 1975).

ANTIOXIDANTS

The lung contains a large variety of both intra- and extracellular antioxidants (Cross *et al*, 1987) among the most important being SOD, catalase and GSH. An imbalance between oxidants and anti-oxidants may result in tissue injury (Halliwell, 1991) and this could well occur in smokers since cigarette smoke contains 10^{16} oxidant molecules per puff (Pryor, 1981) of which 10^{14} are oxygen radicals.

GSH

The concentration of the antioxidant, GSH, which is present both extracellularly, in the lung ELF (Cantin *et al*, 1987) and intracellularly in the type II epithelial cell (Kang & Enger, 1990), is increased two fold in the ELF of chronic cigarette smokers (Cantin *et al*, 1987). This suggests

an adaptation in the form of an enhancement of the lung's anti-oxidant system to cope with an increased oxidant stress. This phenomenon has also been demonstrated in the erythrocytes of cigarette smokers which contain, on average, 26% more GSH than those of non-smokers, and thus have an enhanced ability to protect cultured endothelial cells from oxidative stress (Toth *et al*, 1986). When added in physiological concentrations to isolated epithelial cells treated with CSC, GSH conferred protection as shown by the decrease in cell detachment and the abolition of cell lysis. This was also true for cells treated with the amino acid precursor cysteine or its' acetylated derivative NAC. Exogenous NAC was found to protect against CSC-induced A549 epithelial cell injury, which concurs with the work of Moldéus *et al*, (1986), who reported protection of lung fibroblasts and epithelial cells by NAC(10mM) when exposed to tobacco smoke condensate. NAC has also been shown to protect endothelial cells against oxidative damage (Junod *et al*, 1987). In the system of the isolated, perfused rat lung NAC protects against the cigarette smoke-induced decrease in the lung GSH levels (Moldéus *et al*, 1986; Joshi *et al*, 1988). Rogers & Jeffery (1986) found that heavy exposure of rats to cigarette smoke produced a thickening of the epithelium and an increase in the number of secretory cells in the lung, both of which could be inhibited by the concomitant addition of NAC (1%) into the rat's drinking water.

The protective effects of GSH and other oxidants on aqueous CSC-induced A549 epithelial injury was not observed in experiments where cells were treated with organic CSC. However, organic CSC was prepared in DMSO, a hydroxyl radical scavenger which confers protection on bovine pulmonary artery endothelial cells against endotoxin-induced cytotoxicity (Brigham *et al*, 1987). Although it may be

presumed that DMSO would ameliorate the effects of the particles that make up the organic CSC this was not the case in the experience of this thesis. It may be the combination of the DMSO, which alone at higher concentrations was toxic to A549 epithelial cells, and particles of the CSC that is injurious. Another explanation is that organic CSC injures epithelial cells by a mechanism which is not oxidant-mediated injury. Both Bridges (1985) and Green (1968) found that susceptibility to cell injury by cigarette smoke was significantly correlated with cellular glutathione content. Pre-incubation of the A549 epithelial cells with either cysteine or NAC conferred protection against CSC induced cell damage. This was thought to result from the uptake of these compounds into the cells and subsequent increase in synthesis of GSH thereby increasing the intracellular antioxidant potential (Berggren *et al*, 1984; De Flora *et al*, 1985). However, it should be noted that the intracellular GSH content was not measured in these experiments but should be the aim of future studies. Pre-incubation of the A549 epithelial cells with GSH did not, however, confer protection against CSC-induced injury. Hagen and colleagues (1986) observed that incubation of RTTC with 1mM GSH for 30 minutes prior to the addition of paraquat, which causes a free radical-mediated injury, protected against cell injury; pre-incubation of the RTTC with the amino acid precursors of glutathione, cysteine and glycine, at the same concentration of 1mM had the same effect. These authors suggested that RTTC were capable of taking up GSH. Unfortunately, these GSH pre-incubated cells were not tested for their ability to protect against paraquat toxicity so it is not known whether or not pre-incubation of cells with GSH would produce high enough levels for protection against oxidative stress to occur. The A549 epithelial cells used in this thesis were incubated for 18 hours with GSH, a treatment

shown to have no effect on GSH levels; this contrasts with a 30 minutes incubation which does protect (Dr. I. Rahman, Rayne Lab. personal communication). Taken together these results suggest that a proportion of the effects of cigarette smoke are oxidant-mediated.

BALF failed to protect against CSC-induced A549 epithelial cell injury. This could be explained by the fact that BALF is a mixture of the components of the epithelial lining fluid diluted with a large volume of saline. To observe the true protective potential of these components it would have been necessary to concentrate the BALF. This could be achieved using a variety of methods although each has its disadvantages and may only recover certain portions of BALF components (Reviewed, Klech & Pohl, 1989). These are: (1) pressure filtration, with subsequent loss of 20-50% of total proteins (Lam, 1985); (2) chemical extraction for lipids, with the problem of oxidation; (3) lyophilization which also concentrates the salts present in the fluid and needs to be used in combination with (4) osmotic dialysis, again with protein loss or changes in protein structure. All the above methods were far from ideal and because the composition of the final solution would remain unknown, this line of investigation was not pursued any further.

Although there is a persistent increase in GSH in epithelial lining fluid and erythrocytes in chronic cigarette smokers (Toth *et al*, 1986; Cantin *et al*, 1987), sufficient to protect against the *in vitro* effects of smoke, acute exposure to cigarette smoke has the opposite effect. GSH decreased by 70% in the isolated perfused rat lung exposed to cigarette smoke (Moldéus *et al*, 1986). A 40% decrease in rat lung GSH can also be produced by treatment with BSO, an inhibitor of α -glutamyl cysteine synthetase (Coursin & Cihla, 1988) and BSO has also been shown to decrease GSH levels in A549 epithelial cells (Kang & Enger, 1990).

Endothelial cell cultures treated with BSO have increased susceptibility to the lysis induced by H_2O_2 (Harlan *et al*, 1984). In the experiments in this thesis there was an increased susceptibility to smoke-induced detachment injury when A549 epithelial cells were pre-treated with BSO, adding further support for the role of GSH in defending the lungs against smoke-induced oxidant injury.

As for all pharmaceuticals, BSO should be used in non-toxic concentrations. Mårtensson *et al* (1989) also reduced the GSH levels in murine lung by repeated intraperitoneal injection of 4 m/mol kg^{-1} twice a day for 21 days. Coursin & Cihla (1988) treated rats with BSO at 5m mol. kg^{-1} once or twice a day for a short time course of 4 days. Mårtensson and co-workers reported swelling and disintegration of the type II pneumocyte lamellar bodies and degeneration of the mitochondria, which meant the cell was compromised before any oxidative stress could be added. They went on to argue that the damage done to the lamellar bodies by BSO, shows the need for GSH in maintaining structure/functional integrity. Coursin & Cihla (1988) reported no apparent structural damage with their dosing regime.

SPECIFIC ANTIOXIDANTS

Other important enzymatic anti-oxidants in the lung are the superoxide anion scavenger, SOD, and catalase, which scavenges hydrogen peroxide. In this study the addition of extracellular SOD and catalase to epithelial cells in culture gave no protection against the cell detachment induced by whole CSC although it did prevent cell lysis when the A549 epithelial cell cultures were exposed to vapour phase CSC. However, the intracellular concentrations of these anti-oxidants may be more important in preventing oxidant injury to cells. McCusker & Hoidal (1990) found that the

activities of SOD and catalase in alveolar macrophages were higher in cigarette smokers than in non-smokers. Furthermore, the increased activities of SOD and catalase in the lungs of hamsters exposed to smoke returned to the same level as that of control animals after cessation of the smoke exposure (McCusker & Hoidal, 1990). Both SOD and catalase are unable to penetrate cell membranes in epithelial type II cell culture but the levels of these enzymes can be enhanced by the addition of SOD and catalase encapsulated in liposomes or covalently linked to monomethoxy-polyethylene glycol (PEG). Walther and colleagues (1991) found that increasing both intracellular SOD and catalase, by the addition of PEG-SOD and PEG-catalase, induced an increased resistance to oxidant stress in isolated alveolar type II cells. The lack of penetration of SOD and catalase into cell membranes may at least partly account for the lack of a protective effect of these anti-oxidants against the effects of CSC shown here. Hobson *et al*, (1991), on exposure of rat tracheal explants to varying amounts of smoke for 10 minutes, found focal dose-related damage characterised by blebbing of the apical surface, loss of cilia, and focal cell necrosis. Catalase addition prevented this cell damage as did superoxide dismutase. The authors showed that exposure of tracheal explants to cigarette smoke *in vitro* was associated with histochemical evidence of continuing production of both hydrogen peroxide and superoxide anion at the apical cell membrane. In earlier publications (Churg *et al*, 1989), this group have demonstrated an increase in asbestos fibre penetration of the epithelium of rat tracheal explants following exposure to cigarette smoke and amelioration on the addition of active SOD, catalase or desferrioxamine. Again, this is indicative of an oxygen radical-generated cell injury with the involvement of $O_2^{\cdot-}$, H_2O_2 and $\cdot OH$. One of the most highly reactive oxidants formed *in vivo* is $\cdot OH$, which

can be inhibited experimentally by Desferrioxamine. Pre-incubation of bovine pulmonary endothelial cells with 0.4 nM desferrioxamine for 1 hour significantly protected against injury caused with 50 μ M H₂O₂ (Rinaldo & Gorry, 1990). Rat alveolar type II cells have also been protected against paraquat-induced free radical injury by desferrioxamine (van Der Wal *et al*, 1992). The protection of the A549 epithelial cells against CSC injury by desferrioxamine demonstrated in this thesis again suggested at least a partial \cdot OH oxidant-mediated injury. Cigarette smoke is known to contain at least 6000 different compounds. Among these are toxic electrophilic and reactive compounds, as well as reactive oxygen species (Falk, 1977). Thus it is unlikely that specific scavengers of reactive oxygen intermediates would give total protection against injury caused by CSC.

ANTIPROTEINASE

The antiproteinase α -1PI was partially successful in protecting the A549 epithelial cells against CSC induced damage when incubated with the cells at the same time as the insult. Earlier work of Carp & Janoff (1978) found that freshly prepared aqueous solutions of cigarette smoke suppressed the elastase-inhibiting capacity of human serum. On immunoelectrophoresis of mixtures the aqueous smoke solution, human serum and pancreatic elastase, there was a decrease in elastase/ α ₁-proteinase inhibitor complexes and increased free active protease. Their research went on to suggest that the α -1PI in the lungs of smokers was deficient in its ability to inhibit elastase (Carp *et al*, 1982), although others had been unable to confirm this functional defect in α -1PI (Wyss *et al*, 1984; Abboud *et al*, 1985). Recent work has come out in favour of cigarette smoke inhibiting the functional ability of α -1PI (Ogushi *et al*,

1991). Inactivation of α -1PI in vitro has been shown to be based on the specific oxidation of the reactive-centre methionyl residue. A genetically engineered mutant of α -1PI, containing valine in place of methionine at this position, but otherwise identical to the native inhibitor, is resistant to the effects of vapour phase smoke (Janoff *et al*, 1986). It is likely the protective effect of α -1PI observed in this thesis was due to a non-specific protein effect of the SH groups present in certain amino-acids mopping up some of the free radical produced in the CSC.

MORPHOLOGIC CHANGES

Morphologically cells exposed to cigarette smoke showed cell surface blebbing which would support an oxidant mediated effect of cigarette smoke, since such shape change occurs in other cells following exposure to oxidants (Hyslop *et al*, 1986). This cell surface blebbing or zeinosis occurred upon exposure of the A549 epithelial cells to just one puff of cigarette smoke. Other cells, alveolar macrophages exposed to cigarette smoke (Rasp *et al*, 1978) or ozone (Donaldson *et al*, 1993) in vivo or neutrophils exposed to cigarette smoke in vitro (Lannan *et al*, 1992) also undergo the same membrane changes. Cell membrane blebbing has also been observed in the type I pneumocytes after oxidative injury (Hayashi *et al*, 1987). Oxidative stress produced by quinones, a component of cigarette smoke, has also been shown to deplete intracellular glutathione, followed by oxidation of protein sulphydryl groups which in turn leads to disassociation of the cytoskeleton from the plasma membrane (Bellamo & Mirabelli, 1987-88). This results in disruption of the sites on the actin-binding proteins which attach the cytoskeleton to the plasma membrane, resulting in surface blebbing. Oxidant injury also led to an elevation of intracellular free Ca^{2+} and membrane blebbing itself has been associated

with changes in intracellular Ca^{2+} homeostasis (Jewel *et al*, 1982). Cell swelling and membrane blebbing have been thought to be a part of the process of cell death after a variety of injuries (Trump & Mergner, 1974). Tulenko *et al*, (1988) also suggested that chronic cigarette smoke produced reorganisation of the phospholipid bilayer in smooth muscle cell membrane. Apart from blebbing, cigarette smoke injury may also directly affect the cell membrane by decreasing membrane fluidity as has been shown in alveolar macrophages (Hannan *et al*, 1989). This would decrease the cell's ability to spread and therefore decrease its ability to attach.

PREPARATION OF CIGARETTE SMOKE EXTRACTS

Changes in pH of the assay medium on both exposure to cigarette smoke and CSC were monitored because this in itself may cause functional changes in the epithelial cells. Reduction of the pH of solutions in the epithelium lumen to as low as pH 6 has been shown to have no effect on the bioelectric properties of the epithelium, although further reduction to 2.8 altered the trans-epithelial conductance and was paralleled by increased permeability of the epithelium (Stutts *et al*, 1986). A pH of ≤ 2.2 inhibited active ion transport. The baso-lateral surface of the airway epithelium was much more sensitive to acidification, with the abolition of active transport at a pH of 6 (Stutts *et al*, 1986). However, the addition of cigarette smoke did not alter the pH of the medium of cell suspensions in work done by Bridges (1985), which concurs with the findings in this thesis.

It is of course important to be aware of the many different methods of making and using cigarette smoke and its condensates or extracts when comparing results or relating *in vitro* results to those *in vivo*. The

condensates used in the majority of experiments in this thesis were prepared using HBSS with Ca^{2+} and Mg^{2+} . This buffer does not have the added complications associated with Tris buffer or DMSO. In the Carp & Janoff paper in 1978, the aqueous cigarette smoke extract was made by bubbling the smoke of two unfiltered cigarettes through 0.4M Tris-HCl buffer and this was used at a 10% dilution into their assay system. Wyss & Co-Workers (1984) also used Tris buffer with α -1PI present in the buffer, and when the cigarette smoke was bubbled through it there was no loss of elastase-inhibiting capacity. The work of Wyss *et al* was repeated by Pryor & Dooley (1985) who compared this with their method of bubbling cigarette smoke through phosphate buffer containing α -1PI. They found that the Tris buffer actually protected the α -1PI against fast inactivation caused by whole or gas (vapour) phase cigarette smoke, confirming that Tris was an inappropriate buffer to use in the study of oxidative damage caused by cigarette smoke in vitro. Their work also suggested that the timing of the experiment is critical for the end result. At 25°C, when exposed to cigarette smoke through the phosphate buffer and then left to incubate, α -1PI loses activity. Furthermore, this inactivation occurred in two steps:- the initial fast reaction and the secondary inactivation that occurs slowly and continuously over a period of several days. This slow loss of activity is the same as that seen when α -1PI is incubated with aqueous cigarette smoke extracts. Pryor & Dooley (1985) attributed the fast inactivation of the α -1PI to species present in cigarette smoke that are too short-lived to play a role in α -1PI inactivation by smoke extracts. In the presence of Tris slow inactivation of α -1PI did still happen, although the fast inactivation was quenched. The slow inactivation could be related to the redox cycle of reactive species in condensate as suggested by Church &

Pryor (1985). This group have gone on to report the continuous production of H_2O_2 in aqueous smoke tar solution for 24 hours (Nakayama *et al*, 1989) and beyond. DMSO is another solvent used to produce cigarette smoke extract but care must be taken with this compound. This organic compound has the capacity to disrupt the cell membrane and, as was reported in Chapter 3, causes cell injury at high concentrations. As with many compounds, it can also have an opposing effect. In the case of DMSO a protective free radical scavenging potential has been reported (McDevitt *et al*, 1989). Potentially injurious effects have also been reported with GSH. Richards (1991) showed that the addition of GSH to culture medium just prior to addition of soluble cigarette smoke fraction (SCS) protected Clara cells against damage. However, addition of GSH to the water, through which the SCS was collected, made the SCS approximately 4.5 times more damaging. There are other factors which must also be considered in the production of cigarette smoke extract. Both the type and number of cigarettes as well as the volume of the extract medium are all variable between experimental groups. For example, Holden and co-workers (1989) used one cigarette per ml of fluid while Hunninghake & Crystal (1983) used one cigarette in 24mls of medium. Holden went on to dilute down to a 0.01-1% solution, while the latter group used their extract solution at a 10% dilution. Some groups have used unfiltered cigarettes (Carp & Janoff, 1978; Tardif *et al*, 1990) while others have used filtered cigarettes (Pryor & Dooley, 1985). Truly filtered smoke refers to the vapour or gas-phase smoke and is defined experimentally as that part of the smoke that passes through a glass-fibre Cambridge filter reported to remove 99.9% of all particles $\geq 0.1\mu m$ in diameter (Guerin, 1980). Unfiltered smoke could mean either the smoke from ordinary cigarettes, which

although they contain filters allow a large portion of the particles to pass through, or the truly old fashioned cigarettes which do not contain any filter at all.

Matulionis (1984) reviews the problems of experimenting with cigarette smoke, problems which are also relevant to the study of cigarette smoke condensates. The author identifies several potential sources of variation which require careful evaluation and monitoring: (1) smoke generation; (2) smoke delivery systems; and (3) inherent differences in biological responses to smoke between species. All of these would lead to problems in comparison between laboratories. It is difficult to accurately gauge the composition and concentration of the condensate formed in the lungs of smokers. There have been two basic approaches to estimating the actual concentration of condensate in the airspaces of lung. Laurent *et al* (1983) used one cigarette bubbled through 3mls of phosphate buffer or water (3mls is the average volume of surfactant in a 70kg human) and diluted this 1:21 for final reaction, reasoning that this was within physiological limits. This concentration corresponds to 20ml cigarette smoke per 1ml aqueous solution. They further assumed that, if only 10% of an inhaled bolus of smoke reaches the alveoli, then the smoke concentration used by them in vitro would be reached in vivo after 20 puffs of smoke (2 cigarettes worth) onto 3ml of surfactant. All of these calculations were considered by the authors to be an underestimation of the final concentrations of cigarette smoke and condensate in the alveolus. Holden and colleagues (1989) calculated that if the adult human lungs contain 500ml of tissue and 500ml of blood (Staub, 1982), then the smoke from one cigarette exposed to this would give a concentration of one cigarette/1000ml. Their extract was the smoke of one cigarette/ml, so an equivalent exposure would be 0.1% (vol/vol). They argue that the

circulating blood will carry some of the smoke out of the lung to equilibrate with the total blood volume, so less may be present in lung tissue. Conversely, accumulation of smoke products is likely to occur in habitual smokers, so tissue concentration would be higher which is more likely. The extract used in this thesis was based on Holden's work but was used over a greater range because of the uncertainty and lack of hard facts on the concentration and nature of composition of the condensates in the lung. It is likely therefore that the in vivo concentration was bracketed.

The low levels of this condensate used in these studies caused cell injury to the cancer cell line, A549, which by its nature is probably a tougher and more resistant cell than a non-cancerous type II pneumocyte.

Miyashita *et al*, (1990) found that the malignant cells were more resistant to the effects of smoke. The effects of CSC on epithelial cells were more pronounced with freshly isolated RTTC and the BEA-2B cell line than the A549 cell line, which was derived from type II alveolar epithelial cell carcinoma (Lieber *et al*, 1976). So both freshly isolated RTTC and the non carcinogenic cell, BEA-2B, are more susceptible to CSC than the A549 cell line. One study found the concentration of hydrogen peroxide required to kill alveolar epithelial cells was 14 times higher than the concentration required to kill bovine pulmonary artery endothelial cells (Simon *et al*, 1986). The alveolar epithelium thus appears to be more resistant to hyperoxic-induced injury than the lung endothelium (de los Santos *et al*, 1987). The alveolar type II cells however were found to be more sensitive to hyperoxia in vitro than either lung fibroblasts or alveolar macrophages (Housset *et al*, 1991), even though the fibroblasts had the lowest levels of antioxidant enzymes activities and the lowest content in total glutathione. However, under hypoxic conditions total

glutathione content was unchanged in fibroblasts, contrasting with a significant decrease observed in alveolar type II cells.

DETACHMENT INJURY

Various components other than cigarette smoke present in the lung airspaces could lead to cell injury and detachment. Rickard and co-workers (1992) found that exposure of a monolayer of bovine bronchial epithelial cells to trypsin/EDTA or to elastase led to cell detachment, although this was dependent on the length of time the cells were in culture before being treated. The exposure of these cells to zymosan-activated polymorphonucleukocytes (PMN) also led to cell detachment. Brown *et al* (1992) also previously showed that PMA activated PMN had the capability to cause detachment of A549 epithelial cells. Rickard also found it was possible to increase the susceptibility of these bovine bronchial epithelial cells to protease mediated detachment by pre-incubation for 24 hours with a 10% cigarette smoke extract.

When the type I pneumocytes are damaged, the type II cells divide and differentiate into replacement type I cells (Adamson & Bowden, 1974). This proliferative process will require the cell to modulate its attachment during movement to the area of injury, and spreading to change its' morphology. Cigarette smoke exposure *in vitro* was found to inhibit both cell attachment and proliferation and increase detachment of the A549 epithelial cell, which are all functions relevant to the process of epithelial repair (Rickard *et al*, 1991). Thus cigarette smoke has the potential to affect all parts of the growth and repair cycle, not just a single step.

Loss of cellular adhesion has also been observed with alveolar macrophages when exposed to cigarette smoke extract (Low *et al*, 1977), neutrophils with acute cigarette smoke exposure (Selby *et al*, 1992) and

Clara cells after treatment with the water soluble fraction of cigarette smoke (Richards, 1990).

INTEGRINS

The integrity of airspace and other epithelium is dependent on specific cell surface adhesion molecules or integrins (Albelda, 1991). These are a family of glycoproteins which can be cleaved proteolytically (Spertini *et al*, 1991) and will be vulnerable to oxidants. Thus the effects of cigarette smoke on epithelial cell attachment and detachment could be mediated through a direct effect on cell integrins. A549 epithelial cell detachment is partly mediated via the specific tripeptide sequence RGD (arginine-glycine-aspartic acid) which is present in many matrix proteins such as fibronectin (Dierschbacher & Ruoslahti, 1984), laminin and collagen, and is thought to be the specific recognition site for many of the integrins. RGD has been reported to inhibit the attachment of bronchial epithelial cells (Rickard *et al*, 1991).

As described in Chapter 7 it was not possible to fully ascertain whether the A549 epithelial cells which are transferred human type II cells had the same integrin expression as normal human type II pneumocytes *in vivo*. This comparison of the *in vitro* situation to the *in vivo* situation would be valuable, since endothelial cells in culture have been shown to have a different integrin repertoire to endothelial cells *in situ* (Albelda *et al*, 1989). The A549 epithelial cells were, however, partially characterised for the expression of cell surface adhesion molecules, and were found to be positive for CDw49b, (α_2), CDw49e (α_5), CDw49f (α_6), α_v and CD29 (β_1) but were negative for CDw49d (α_4) and $\alpha_v \beta_3$, as were the BEA-2B epithelial cell line. These results concur with other findings (Mette *et al*, 1993), although trace amounts of $\alpha_v \beta_3$ were present on the A549

epithelial cells in their study. A549 cells were also found to qualitatively express a pattern of integrins similar to that of the normal bronchial epithelial cells isolated from fragments of human bronchial tissue obtained from surgical pathology specimens (Mette *et al*, 1993). These isolated cells did not express $\alpha_v\beta_3$, although the α_v sub-unit was detectable with a specific anti- α_v mAb, and this was also true of the A549 epithelial cells examined in this thesis. The α_v on the A549 epithelial cells is probably accompanied by β_5 or β_6 subunit, which have previously been reported to be expressed by epithelial cells (Cheresh *et al*, 1989). Recent reports have demonstrated that the α_v subunit can associate with the β_1 subunit (Bodary & McLean, 1990), the β_5 subunit (Smith *et al*, 1990), the β_6 subunit (Sheppard *et al*, 1990) and the recently described β_8 subunit (Moyle *et al*, 1991). Bretscher also found that the A431 cell line derived from a human squamous epidermal carcinoma expressed $\alpha_5\beta_1$, $\alpha_3\beta_1$ and $\alpha_6\beta_4$ (Bretscher, 1992). The expression of these β subunits on the A549 epithelial cells has still to be assessed. The integrin repertoire of cultured bronchial epithelial cells (Mette *et al*, 1993) was similar to that of other cultured epithelial cells (Klein *et al*, 1990; Kurpakus *et al*, 1991), and the distribution precisely mirrored that found in bronchial epithelial cells in situ (Damjanovich *et al*, 1992). The BEA-2B epithelial cell line is closer in phenotype to the normal bronchial epithelium and, as stated earlier in Chapter 7, also has similar integrin expression to the A549 epithelial cells and therefore also to the normal bronchial epithelium reported by Mette *et al*, (1993). Therefore it is likely that the integrins expressed by the A549 epithelial cells are also those expressed by the type II pneumocytes in situ. The method used by Damjanovich and colleagues for determination of distribution of integrins in normal lung tissue was similar to the method used in Chapter 7. The staining was treated in the same manner with

antibody and detection was with the Vectastain ABC kit. The only difference was that no counterstain was applied, since its omission was meant to increase sensitivity. While the staining of the bronchial tissue was cell-specific, precise identification of individual cell types within the alveolar septae was not possible. The staining for α_1 , α_2 and α_3 at this level suggested that the collagen-laminin receptors were present throughout the endothelial and epithelial layers of the alveoli, with staining for the α_6 receptor being more localised at the basolateral surface of the alveolar epithelium and/or endothelium. The alveolar tissue exhibited weak and diffuse staining patterns with mAbs against α_5 and α_v .

There were a number of reasons why the staining patterns were erratic and non-specific. The first problem was the availability of "normal" tissue, which if fresh is likely to have come from the resected lung of a smoker. Approximately 90% of the operations for resection of lung tissue are carried out on smokers or ex-smokers whose lungs are thus not "normal". The next problem is the method of fixation, if formaldehyde is used for example then the epitopes for certain mAbs are destroyed. The length of time that tissue has been in fixative is also important. If the tissue is in too long then there can be masking or destruction of antigen. This method of fixation allows the tissue to be paraffin embedded which retains normal lung structure but unfortunately certain mAbs do not work on such sections. The alternative is frozen section which is technically more difficult and often leads to distortion or loss of normal lung structure which could lead to difficulty in interpretation. The mAbs themselves are also a problem as they can label fresh cells for functional assays or flow cytometry but will not work on tissue sections. It is therefore necessary to be very careful in the choice of lung tissue,

fixation solutions, embedding compounds and source and type of mAb's. In both Chapters 7 and 8, the problem of autofluorescence of the epithelial cells, due to whole cigarette smoke exposure, was encountered. Several methods have been suggested to overcome the analytical problem caused by autofluorescence (Steinkamp & Stewart, 1986; Alberti *et al*, 1987) based on the differences between the broad autofluorescence emission spectrum and the well-defined spectrum of the fluorochrome label. Unfortunately, the high intensity of the autofluorescent signal, which has been shown to be emitted by alveolar macrophages exposed to cigarette smoke made these methods non-viable. A recent publication has, however, suggested a method for quenching intracellular autofluorescence in alveolar macrophages recovered from cigarette smokers, that permits the analysis of fluorochrome labelled surface antigens by flow cytometry (Halldén *et al*, 1991). This group used a crystal violet solution with permeabilization of the cell membrane with the detergent n-octyl- β -D-glucopyranoside (OG) prior to fixation of the cells for fluorcytometric analysis. Crystal violet was previously shown to quench FITC-conjugated yeast particles (Hed, 1977) which emit high levels of fluorescence. The method of internalisation and concentration of crystal violet used by Halldén was found to be critical. The crystal violet alone decreased viability and the availability of CD11b on alveolar macrophages even at low concentrations. Pre-incubation of the cells with the detergent OG before addition of crystal violet increased cell viability and better preserved the CD11b expression but prevented efficient quenching with the crystal violet. This method has its problems as the authors stated that it was difficult to obtain the same level of quenching with different batches of crystal violet solution prepared from equal amounts of dye; reporting difficulty with the solubilisation of crystal

violet during preparation and after in storage. Although this technique did quench the autofluorescence, how reliable and repeatable it is still needs to be proven. To apply this methodology as it stands, to the epithelial cells in culture exposed to whole cigarette smoke, would not give accurate results that could be compared between experiments.

ECM COMPONENTS

Attachment of cells to ECM plays an important role in the regulation of cell growth and differentiation, both of which are important in repair. With attachment of the cells to individual components of the ECM, there appears to be more than one mechanism or integrin involved in attachment. There also appears to be a variable rate of attachment of cells depending on the individual ECM components. This has been demonstrated by other research groups investigating the attachment properties of bovine bronchial epithelial cells (Rickard *et al*, 1991). The cultured bovine bronchial epithelial cells had the highest percentages of attachment to collagen I and the biosynthesized ECM. The rate of attachment of these cells was more rapid than the A549 epithelial cells with no more attachment occurring after 2 hours of the bovine bronchial epithelial cells, as shown in Chapter 3. The A549 epithelial cells were shown to continue to attach after 4 hours incubation. The BEA-2B epithelial cells were relatively slow at attaching in comparison to the A549 cells and were not used in any detailed experiments of attachment. The A549 epithelial cells that were exposed to either whole or vapour phase cigarette smoke showed decreased ability to attach to each individual ECM component when compared with the control cells attaching to that component. Although there was variability in the level of attachment, greater attachment to collagen I and collagen IV, both of

which are prominent components in the lung and at the alveolar level, was observed. This concept of collagen I and collagen IV being important in maintaining the integrity of the epithelial cell barrier is further borne out in the detachment assay. Cells cultured on collagen I in particular were found to undergo less cell damage than control cells. The damage to the cells was however not completely abolished. The cigarette exposure therefore affects some or all of the integrins involved in attachment.

CYTOSKELETAL EFFECTS

The exposure of epithelial cells to cigarette smoke *in vitro* in this thesis suggests modification in the activity, but not expression, of integrins. It is likely that a direct effect of cigarette smoke on cell surface adhesion molecules may be only part of the mechanism of epithelial cell detachment. These adhesion molecules are thought to interact with the cytoskeletal proteins (Burridge *et al*, 1988). There is biochemical evidence for interaction of β subunit of integrins or cytoplasmic domain peptides with the cytoskeletal proteins talin (Horwitz *et al*, 1986) and α -actinin (Otey *et al*, 1990). There is also indirect evidence for interaction of α subunit cytoplasmic domains with the cytoskeleton. The integrin $\alpha_5\beta_1$ is in clusters or focal contacts, whereas $\alpha_3\beta_1$ is not, although both interact with fibronectin (Elices *et al*, 1991). These results indicate the importance of different integrin subunits mediating differing cellular responses to common extracellular ligands. Although most of this is conjecture built on indirect observation, $\alpha_6\beta_4$ is specifically concentrated at hemidesmosomes in epithelial cells (Kurpakus *et al*, 1991) where it is thought to interact with cytoskeletal intermediate filaments which are associated with hemidesmosomes. More studies need to be carried out on integrin-cytoskeleton association but Hynes (1992) stated that one of

the major functions of integrins is to mediate cytoskeletal interactions at the inner face of the membrane at site of cell-substratum or cell-cell adhesion. Cigarette smoke could therefore effect the function of the integrins by causing conformational changes in the protein or changes in the distribution of the integrins, thereby effecting their interaction with the cytoskeleton, with subsequent loss of cell-substratum or cell-cell adhesion. Other *in vitro* studies have described oxidant-mediated effects on the cytoskeleton of alveolar cells which could culminate in cell detachment (Raghu *et al*, 1984; Welsh *et al*, 1985). Ozone, an oxidant and a component of cigarette smoke, has been shown to disrupt the actin-rich microfilaments and the microtubules of cultured epithelial cells; and at concentrations between 0.8 and 2 ppm O_3 exposed epithelial cells are also rounded and less well spread (Bhalla *et al*, 1990).

Cigarette smoke can effect the cytoskeleton of the cell by altering the F-actin. The change in the actin staining patterns of CSC treated cells compared to control cells was similar to that seen by Holden *et al* (1989). They found the exposure of monolayers of endothelium to cigarette smoke extract (1%) led to a change in actin staining. The control monolayer was seen to have diffuse perinuclear fluorescence representing non-polymerized G-actin. After 15 minutes of exposure to cigarette smoke extract the staining changed, suggesting polymerization of actin filaments which was still diffuse. One hour of exposure to the extracts highlighted gaps between the cells with staining of peripheral actin bands in occasional cells. Intact microfilaments are probably necessary for the preservation of normal cell morphology, cell spreading and the maintenance of intracellular contacts observed in cultured cells (Savion *et al*, 1982; Kaiho & Miso, 1990). If the ability of type II epithelial cell to spread and attach is diminished, this will interfere with epithelial repair

and may be compounded by a decrease in cell proliferation. This process may be relevant to the alveolar destruction which leads to emphysema. In a model of wound repair of human surface respiratory epithelium in vitro, Zahm and colleagues (1991) showed that there were two stages to the re-epithelialization. The first involved cells adjacent to the wound extruding prominent lamellipodia towards the wound, where with cell spreading there was progressive flattening of the microvilli on the lamellipodia with dynamic and directional polymerization of actin filaments. The second process involved sheet migration of the upper layer of cells. Actin staining demonstrated the presence of stress fibres in the migrating cells. Treatment of the wounded culture with cytochalasin B ($5\mu\text{M}$), which blocks actin polymerization, inhibited protrusion formation and cell migration. The sheet migration of epithelial cells would not necessarily occur in the alveoli, but the cell spreading and moving would. The A549 epithelial cells detached when treated with cytochalasins, showing that an intact cytoskeleton is required for normal morphology. Treatment with CSC was shown to change the actin staining patterns of A549 epithelial cells with increased peripheral actin staining. These cells were shown to round up and decrease in the area. All of these would be detrimental to the cell spreading required for repair of denuded epithelium in a damaged alveoli. Bhalla *et al* (1990) reported that the microtubule inhibitor, vinblastine, did not cause increased permeability in a confluent monolayer of rat epithelial cells. Colchicine, the microtubule inhibitor used in this thesis did not prevent epithelial wound repair in another study (Zahm *et al*, 1991). So an absence of effect of colchicine on either A549 epithelial cell detachment or cell lysis is not surprising.

The simple idea of the cell surface adhesion molecule "glueing" the cell

to the subtraction, or to another cell, has long since been abandoned in favour of a very much more complex picture (Hynes, 1992). The changes which occur in the interaction of integrins and the cell cytoskeleton may be a central component in the processes which enable cells to respond to external signals. The probability is that the cigarette smoke will both directly and indirectly affect integrins by a series of activation and inactivation reactions caused by cell signalling.

CELL SIGNALLING

Cell signalling occurs in response to a change in the external environment of a cell. The inhalation of cigarette smoke changes the environment of the pneumocytes in the lung and a percentage of the smoke's components are soluble, allowing access to the membrane and thereby to the interior of the cell. Stimulation of the cell-surface receptors initiates hydrolysis of a membrane-bound inositol lipid, which produces at least two second messengers - diacylglycerol and inositol 1,4,5-trisphosphate [$\text{Ins}(1,4,5)\text{P}_3$]. These messengers are generated by a membrane transduction process comprising three main components: a receptor, a coupling G protein and phosphoinositase C. The diacylglycerol acts by stimulating PKC, whereas $\text{Ins}(1,4,5)\text{P}_3$ releases calcium from internal stores. These pathways form the cornerstone of an ubiquitous transduction mechanism which regulates a large array of cellular processes. Pertussis toxin which has the ability to block some, but not all, of the G-proteins responsible for signal transduction, decreased cell lysis caused by vapour CSC, although there was a subsequent increase in cell detachment. This implied that cell lysis was the result of cell signalling through the cell membrane and could be due either to a stimulation in PKC or increased $[\text{Ca}^{2+}]_i$.

Conflicting results, described in Chapter 9, were experienced with manipulation of protein kinase A, using cAMP or forskolin, and protein kinase C, using staurosporine and calphostin C. Dibutyl cAMP at 10^{-3}M has been reported to cause a significant increase in ciliary beat frequency of human respiratory epithelium (Di Benedetto *et al*, 1991), and at the same concentration cause a significant increase in A549 epithelial cell lysis on exposure to vapour CSC. This concentration may seem relatively high, but is required to induce a change in intracellular protein phosphorylation (Cohen, 1987). In the macrophage Hamachi *et al* (1984) have shown the Bt_2 cAMP promotes F-actin disassembly and inhibits actin polymerisation, while Petty & Martin (1989) have shown that well spread flattened macrophages treated with Bt_2 cAMP round up and are not as well attached. Furthermore, these authors also show changes in the F-actin staining patterns of treated cells typified by a circular cortical layer around cell periphery. Increasing cAMP, using forskolin or a cAMP analogue in human endothelial cells also disrupted actin microfilaments and caused the same peripheral actin staining (Lampugnani *et al*, 1990). The treated cells still had the ability to attach although did not spread well and those treated with forskolin were subsequently found to be more resistant to proteolytic detachment. A549 epithelial cells treated with forskolin were found to be more resistant to the damaging effects of CSC. Thus both Bt_2 cAMP and forskolin disrupt microfilaments in the cell and inhibit cell spreading but forskolin must affect certain integrins or adhesion mechanisms to account for the tighter attachment. The older PKC inhibitors, of which staurosporine is one, are less specific in their actions than the newer ones, of which calphostin C is one. Staurosporine has been reported to induce dissolution of the microfilament bundles in intact cells, independent of protein kinase C (Hedberg *et al*, 1990). This

could lead to cell detachment as disruption of the microfilaments with cytochalasin caused A549 epithelial cell detachment. Staurosporine, like phorbol esters and diacylglycerols, is also known to induce the translocation of PKC to membranes (Wolf & Baggiolini, 1988) which is more indicative of activation than inhibition. The inhibiting capacity of calphostin C has been reported to be highly specific for protein kinase C, with interaction with the protein's regulatory domain (Kobayashi *et al*, 1989).

Elucidation of the mechanisms of A549 epithelial cell detachment showed that activation of PKC had a role and that part of the cigarette smoke-induced cell injury was also due to activation of PKC. Miyashita and co-workers (1990) found that treatment of normal human bronchial cells (NHBC) with CSC reduced their ability to bind PDBu, the PKC activator. Whether this effect was due to direct competition inhibition, reduced binding affinity, or activation of phospholipase C was unknown. Pre-incubation of the A549 epithelial cells with calphostin C reduced cell injury, suggesting a role for PKC in this injury. Both H-7 and H-8 are more general inhibitors of the protein kinases. H-8 also reduced CSC induced cell injury again supporting the role of PKC. H-7 did not ameliorate the injury and this inhibitor may need to be used at higher concentrations, as Di Benedetto *et al* (1991) found that H-7 was inhibitory to respiratory epithelial cells at 100 μ M but not 10 μ M.

It is possible that oxidant-mediated increases in intracellular calcium may be the mechanism involved in the changes in cell function and structure. Use of ionomycin to increase the $[Ca^{2+}]_i$ levels in A549 epithelial cells lead to a time and dose-dependent cell injury with cell detachment followed by cell lysis. These effects were also shown with higher concentrations of the calcium ionophore A23187. Winter *et al*(1991)

failed to find an increase in Madin Darby canine kidney (MDCK) epithelial cell permeability with A23187 at concentrations of 1, 2 and 5 $\mu\text{g} \cdot \text{ml}^{-1}$ for 1 hour of treatment; this is in agreement with the work in this thesis which showed no cell injury at 1 and 2 μM after 1 hour of treatment. Using Madin Darby canine kidney (MDCK) epithelial cells, 50 μM A23187 rapidly increased permeability of monolayers (Martinez-Palomo *et al*, 1980), and at this concentration A549 epithelial cells underwent lysis.

Using cation chelators it was possible to virtually abolish cell detachment and cell lysis caused by CSC. Holden *et al* (1989) found that the presence of extracellular Ca^{2+} and Mg^{2+} was necessary to increase the flux of ^{125}I -albumin across endothelial monolayer treated with cigarette smoke extracts. Absence of cations abolished the increased permeability across the endothelial cell monolayer. Rojanasakul *et al* (1993) found that addition of a hydroxyl radical-generating system to alveolar macrophages caused an initial rapid rise in $[\text{Ca}^{2+}]_i$, followed by a slower additional elevation of $[\text{Ca}^{2+}]_i$ and these alterations preceded the depletion of ATP. Both depletion of extracellular Ca^{2+} and pre-incubation of cells with a Ca^{2+} channel blocker inhibited $[\text{Ca}^{2+}]_i$ response and cell injury. Incubation of A549 epithelial cells with EDTA or EGTA inhibited the cell injury caused by CSC.

An increase in $[\text{Ca}^{2+}]_i$ caused by cigarette smoke could potentially mediate several Ca^{2+} dependent cellular events leading to the degradation of membrane phospholipids, and cytoskeletal and membrane proteins. Ca^{2+} is required for a number of active processes in the cell and therefore CSC is likely to be acting through a number of pathways. It has been suggested that oxidation of membrane lipids may allow them to function as calcium ionophores, thereby increasing intracellular calcium (Serhan *et*

al, 1981). Influx of Ca^{2+} is known to be associated with actin filament contraction in endothelium (Shasby *et al*, 1985).

As well as elevation of $[\text{Ca}^{2+}]_i$, oxidant injury to cells leads to a rapid loss of cellular adenosine triphosphate (ATP), which is a catalyst for many biological processes. Steady-state concentrations of ATP provide cells with sensitive mechanisms to control and regulate functional activities, and a balance exists between energy-utilizing and energy-generating processes. Both the ATP-generating and the ATP-requiring processes can be slowed or accelerated in the normally functioning cell when conditions in the cell change. Acute exposure to cigarette smoke has been shown to decrease cellular ATP content (Voisin *et al*, 1985), as has injury to endothelial cells in culture caused by exposure to 5mM H_2O_2 (Spragg *et al*, 1985). Depletion of ATP also occurred with the addition of 1 or 2 mM H_2O_2 to MDCK cells. Specific depletion of ATP using 2-deoxy-P-glucose (DOE) down to levels produced by the oxidant did not change the cell monolayer permeability (Winter *et al*, 1990). These observations are consistent with the idea that oxidant-stimulation of diglyceride production with consequent stimulation of protein kinase C, is one mechanism by which oxidants can alter epithelial cell permeability. Diglycerides are important messenger molecules that activate protein kinase C in many cell types (Henson & Johnston, 1987).

Depletion of ATP is correlated with progressive disruption of microfilament architecture in both endothelial (Hinshaw *et al*, 1993) and epithelial cells (Molitoris *et al*, 1991). In adherent endothelial cells discrete stages in the process of ATP-dependent disruption and reassembly of microfilaments were identified. Initially there is thinning of the stress fibres and increased staining of peripheral microfilaments, proceeding to fragmentation of the filaments into many shorter segments.

Actin polymerization accompanies this process because fragmentation will lead to the presence of increased actin nucleation activity.

PROLIFERATION

The demonstration that CSC has a detrimental effect on the proliferative potential of A549 epithelial cells concurs with the work of Miyashita and co-workers (1990), who reported that cigarette smoke inhibited the proliferation of both normal and malignant human bronchial epithelial cells. Rat type II cells, when exposed to 95% O₂ for 24 hours, were shown to have decreased thymidine incorporation into DNA (Housset *et al*, 1991). Severe hyperoxia injury has been reported to lead to retarded repair of the alveolar epithelium, with disturbance to normal epithelial-fibroblast interaction sufficient to promote the fibrotic process (Adamson *et al*, 1988).

If cigarette smoke damages the epithelium then why does pulmonary fibrosis not occur in every case? As previously mentioned, in the acute phase of cigarette exposure there is a drop in the lung GSH levels, but chronic cigarette smokers have twice the normal levels of GSH (Cantin *et al*, 1987) and increased levels of certain other antioxidants. (McCusker & Hoidal, 1990). The extracellular levels of GSH in the lung have been implicated in the control of fibroblast proliferation. High levels of GSH (500 μ M) have been reported to suppress fibroblast proliferation in vitro (Cantin *et al*, 1990), while patients with the fibrotic lung disease, idiopathic pulmonary fibrosis (IPF), have low levels of ELF GSH (Cantin *et al*, 1989).

The proliferative capacity of the A549 epithelial cells was shown to be protected by extracellular GSH in physiological concentrations. Similarly, intracellular reduced glutathione has been found to be important

in cell proliferation (Kang & Enger, 1990). A reduction in intracellular GSH levels with BSO treatment results in inhibition of A549 epithelial cell division. Since cigarette smoke has been shown to decrease the intracellular levels of the A549 epithelial cell GSH, this may also contribute to the effect of CSC on epithelial cell proliferation.

INFLAMMATORY MEDIATORS

In the detachment assay system neither TNF nor LPS were found to cause detachable cell injury. These compounds need to bind to a receptor to activate changes within a cell. The A549 epithelial cells may not process the receptors required, although this is unlikely in the case of TNF. The A549 epithelial cell when incubated with TNF expressed mRNA for Interlukin-8 (IL-8) in both a dose and time-dependent fashion (Standiford *et al*, 1990). Furthermore, increased permeability of kidney epithelial cells *in vitro* occurred with 40-59 mg ml⁻¹ TNF (Mullin & Snock, 1990). Monolayers of bovine pulmonary arterial endothelial cells also showed an increase in albumin flux *in vitro* after treatment with TNF, but when measured for its cytotoxic effects by ⁵¹Cr release from the endothelial cell monolayer was found to cause no significant cytotoxic effects (Goldblum & Sun, 1990). This concurs with the work in Chapter 3 showing no increases in cell detachment or cell lysis. Although Marcho *et al*(1991) found exposure of their bovine pulmonary artery endothelial cell to TNF- α resulted in a dose-dependent reduction in cell viability. TNF has generally been described to cause increased permeability of cell monolayers and there are varying reports as stated above on the cytostatic or cytotoxic effects of the cytokine. These effects are dependent on the cell type, dose of TNF, duration and conditions of exposure. Marcho *et al* (1991) also reported that TNF caused distinct morphologic changes

with a redistribution of the actin filament and micrographs showed increased peripheral actin staining with rhodamine-phalloidin in TNF-treated endothelial cells. Pre-treatment of these cells with TNF for 18 hours reduced intracellular GSH concentrations, and were subsequently more susceptible to H_2O_2 mediated cytotoxicity. This concurs with the earlier reported work of Rickard and co-workers (1992) where incubation of epithelial cells with sublethal levels of cigarette smoke extract, which is known to reduce intracellular GSH levels, increased susceptibility to protease. The exact mechanism of TNF induced cytotoxicity is unclear although increasing evidence suggests that one such mechanism may involve the production of reactive oxygen species. TNF-treated tumour cells and normal cells have recently been reported to generate O_2^- and H_2O_2 (Meier *et al*, 1989). GSH depletion and actin filament redistribution, which are common to both TNF and cigarette smoke exposure, may well be a general reaction of the cell to oxidant stress.

The *E. coli* endotoxin (LPS) has been reported to cause morphologic changes of type I pneumocytes 60 minutes after intravenous infusion into anesthetized sheep, with a few regions in the alveoli showing disruption and loss of type I pneumocytes (Meyrick & Brigham, 1983). Wiener-Kronish *et al*, (1991) however described contradictory results with no airway epithelial cell changes following instilled *E. coli* endotoxin in sheep. In both cases however there was an inflammatory response.

In summary this thesis has shown that cigarette smoke has a detrimental effect on cultured epithelial cells, causing cell lysis, detachment and reduction in ability to attach and also to accumulate [^3H]. These effects of cigarette smoke are probably oxidant-mediated although the exact mechanism has not been elucidated. These effects, which have important implications for epithelial injury and repair *in vivo* following smoke

exposure, can be ameliorated by the presence of GSH in concentrations present in the epithelial lining fluid of chronic smokers. It remains to be proven whether repeated oxidative insult from acute smoking could result in a temporary local GSH deficiency in the lung airspaces resulting in direct oxidative injury to airway epithelium.

To examine the earliest effects of cigarette smoke on the epithelium mimicking the *in vivo* situation as closely as possible, monolayers of epithelial cells should be exposed to cigarette smoke and not condensate, which is a secondary product, although no less important. To achieve this aim an exposure system has been commissioned within our laboratory, based on that of Valentine (1985), where a rotating platform is inclined to alternately expose cells to culture media and cigarette smoke. This is only now available for use but too late for this thesis work.

To understand the complexity of lung disease it is necessary to be aware of the fact that each smoker is an individual and as such will react uniquely to the insult of cigarette smoke. If this was not the case then every smoker would develop lung cancer or have the classical tissue destruction that manifests as emphysema.

How cigarette smoke affects the lungs in each individual is probably dependent on their antioxidant status and their cells' ability to carry out biochemical detoxification. The respiratory tract is capable of producing large numbers of metabolites from inhaled parent chemicals (Cohen, 1990). At the alveolar level the type II cells contain a rich supply of phase I and phase II components for biotransformations (Dahl & Hadley, 1991). The classical phase I enzymes are the cytochrome P450 monooxygenases while the phase II reactions are catalysed principally by the glutathione S transferase isozymes which catalyze the interactions between GSH and metabolites. This combination of enzymes could lead

to metabolic activation and then detoxification of certain compounds. It is the balance between these processes that is important in biotransformation. Less severe injury to the epithelium leads to normal repair. It is possible that cigarette smoke inhalation could cause small localized areas of injury with the loss of a few epithelial cells which then leave the interstitium open to damage and subsequent loss of the connective tissue. Although the effects of cigarette smoke on the connective tissue of the lung have not been examined in this thesis, it is important to remember that cigarette smoke has the potential to degrade hyaluronic acid in vitro (McDevitt *et al*, 1989) and in vivo (Konno *et al*, 1982). The water-soluble components of the vapour phase cigarette smoke have been shown to inhibit formation of desmosine cross-links and suppress oxidative deamination of tropoelastin by purified lysyl oxidase in vitro, thereby interfering with elastin cross-linking (Laurent *et al*, 1983). These studies were extrapolated in vivo by demonstrating that cigarette smoke impairs elastin resynthesis in lungs of hamsters with elastase-induced emphysema (Osman *et al*, 1985). This taken together with the possible loss of alveolar epithelium may exacerbate alveolar destruction. After years of this minimal but gradual destruction the process of emphysema would become apparent.

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